View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Carolina Digital Repository

# SEARCHING FOR NEW PERIODONTAL PATHOGENS: INSIGHTS FROM AN ENHANCED RNA-OLIGONUCLEOTIDE QUANTIFICATION TECHNIQUE (ROQT)

Bruno Schneider Herrera

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Periodontology, School of Dentistry

Chapel Hill
2018

Approved by:

Flavia Teles

Steven Offenbacher

Julie Marchesan

©2018
Bruno Schneider Herrera
ALL RIGHTS RESERVED

## ABSTRACT

Bruno Schneider Herrera: Searching for New Periodontal Pathogens: Insights from an Enhanced RNA-Oligonucleotide Quantification Technique (ROQT)
(Under the direction of Flavia Teles, Steven Offenbacher, and Julie Marchesan).

*Aims*: Fifty percent of the oral microbiome remains unrecognized or uncultured. In order to study this segment of the microbiota, we previously developed the RNA-Oligonucleotide Quantification Technique (ROQT). The aim of this study was to optimize the ROQT technique regarding its sensitivity, specificity and cost-effectiveness. *Material and Methods*: Total nucleic acids (TNA) were extracted from bacterial suspensions and subgingival biofilm samples using manual and an automated protocol. RNA, DNA and Locked Nucleic Acid (LNA) digoxigenin-labeled oligonucleotide probes targeting 21 cultured/uncultured taxa were synthesized. Tests were performed using 10ng TNA, $10^6$ bacterial cells, and RNA and DNA standards for quantification. Probe specificity was determined by targeting 96 oral bacterial species; sensitivity was assessed using serial dilutions of reference bacterial strains. The tested conditions were assessed in a small pilot study with subgingival biofilm samples. Significance of differences between test conditions and subject groups was determined using the Mann–Whitney U-test. *Results*: LNA-oligunucleotides probes yielded stronger signals without cross-reactions, when compared with DNA and RNA oligonucleotides probes. The automated method at 63C consistently yields stronger signals in comparison to the manual protocol. Samples from patients with periodontitis showed higher levels of subgingival bacteria based on the universal probe signals than samples from periodontally healthy subjects. Overall, the most commonly detected

uncultivated/unrecognized species in the samples from severe sites were probe *Treponema sp* ot254, *TM7* ot356, *Fretibacterium sp* ot360, Y73 *Selenomonas sp* ot134, 54, *and Desulfobulbus* sp. ot041. In the cultivated segment of the microbiota, the most abundant taxa were *Tannerella forsythia* ot613, L66 *Oribacterium sp* ot78, K76 *Bacterioidetes sp* ot274*, Fretibacterium fastidiosum* ot363 and *Porphyromonas gingivalis* ot619. *Conclusion*: The use of automated TNA extraction, LNA probes and RNA standards enhances the sensitivity, specificity, throughput, and cost-effectiveness of ROQT.

## ACKNOWLEDGEMENTS

I would like to acknowledge my mentor, Dr. Flavia Teles, for her wisdom, guidance, encouragement throughout the completion of my research project. I sincerely appreciate your kindness and selfless support; you definitely went above and beyond to help me throughout this process.

Lynn Martin, Dr. Sandra L. Henz, and Shawn Dua for all their dedication and hard work in this project.

My family and friends for all their love and support over the years.

My co-residents, Brenda, Chip, and Megumi for all their support and friendship during my residency.

Lastly, to Dr Ricardo Teles, who has been my role model and friend. I have been fortunate to work with great researchers, taught by great faculty and shared the clinic with great periodontist, but few people out there can inspire others like Dr. Teles. I am a better professional and I am a better human being because Dr. Ricardo Teles took the time to challenge my assumptions and mentor me along the way.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| BOP | Bleeding on Probing |
| CAL | Clinical Attachment Loss |
| CEJ | Cemento-Enamel Junction |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediamine Tetraacetic Acid |
| GCF | Gingival Crevicular Fluid |
| $H_2O_2$ | Hydrogen Peroxide |
| HCl | Hydrogen Chloride |
| HOMIM | Human Oral Microbial Identification Microarray |
| HOT | Human Oral Taxa |
| IL-1β | Interleukin 1β |
| LNA | Locked Nucleic Acid |
| mM | Milimolar |
| NaCl | Sodium Chloride |
| NIDCR | National Institute of Dental and Craniofacial Research |
| NIH | National Institute of Health |
| PCR | Polymerase chain reaction |
| PD | Probing Depth |
| pM | Picomolar |
| QCS | Quality Control Sample |
| RNA | Ribonucleic Acid |
| ROQT | RNA-Oligonucleotide Quantification Technique |

| | |
|---|---|
| SDS | Sodium Dodecyl Sulfate |
| SSC | Saline Sodium Citrate |
| UV | Ultraviolet |

## INTRODUCTION

The oral cavity harbors a complex microbiome, with close to 700 taxa capable of colonizing the oral cavity[1]. Most of the current knowledge regarding the microbial aspects of periodontal health and disease, as well as changes in the microbiome that result from periodontal treatment are based on the cultivated segment of the microbiome, largely guided by the seminal work of Socransky et al (1998)[2] on the subgingival microbial complexes. However, there is a substantial portion of the oral microbiome that has not yet been fully characterized or has remained uncultured[1].

Although the role of the unrecognized/uncultivated organisms in periodontal health and disease is currently unknown, there is no reason to expect that this segment of the microbiota harbors fewer pathogens than its cultivated counterpart. In fact, it is possible that a red complex "equivalent" might exist among unrecognized and uncultivated taxa. In that scenario, it becomes crucial to study them in detail, so that their pathogenic properties can be unveiled. However, the first challenge in that process is to determine which organisms are relevant to health maintenance and for disease initiation. There are currently 94 unrecognized taxa and 208 uncultured phylotypes described in the Human Oral Microbiome Database (www.homd.org[3]) it is unlikely that they are all equally important in the pathogenesis of periodontitis.

In order to select which of those taxa merit further study, one needs to determine whether the taxa presence levels are likely to have a role in the dysbiotic environment that precedes disease initiation. With that in mind, in 2011, Teles et al.[4] developed the RNA-Oligonucleotide

Quantification Technique, a cost-effective approach to enumerate uncultivated/unrecognized taxa in individual samples of subgingival biofilm samples[4,5]. ROQT can overcome limitations of other techniques that require sample dilution, sample pooling or PCR amplification[6–10], all of which can add bias to the microbial results. In addition, it provides quantification, a key piece of information in the study of microbial shifts in periodontal health and disease, as the differences between periodontal health and disease and before and after therapy are quantitative, rather than qualitative[2,11]. The utility of ROQT has been demonstrated in several papers that have helped identify new candidate periodontal pathogens[12,13].

But first, we will give an overview about the biology of the biofilm, its ecological relationship, its role in the periodontal disease, and the new pathogens that will be targets for this and for future research.

## REVIEW OF THE LITERATURE

### BIOLOGY OF THE BIOFILM

Among the advances in microbiology that have taken place over the past 50 years, the study of how microbial growth and develop on surfaces in complex communities has been one of the subtlest.

Definition of biofilm: Biofilm is embedded in a matrix of extracellular polymeric substances and is a mode of microbial growth where dynamic communities of interacting sessile cells that can be detached and colonize other surfaces[14].

Many different biofilms exist in nature; some are useful (to the human), and others are associated with potentially harmful effects. Dental plaque is a naturally-occurring biofilm that has the potential to cause disease. Dental plaques have many properties in common with biofilms found in other locations. However, they have certain characteristics that are important in terms of the development and control of the periodontal diseases[15].

The features, structure and the basic components of biofilms: There are four characteristics of biofilm: autopoiesis (the ability to self-organize), homeostasis (to resist environmental perturbations that results in new homeostatic states), synergy (must be more effective in association than in isolation) and communality (should respond to environmental changes as a unit rather than as single individuals)[15].

In a biofilm, residing bacteria are protected from environmental threats; trapping of nutrients and metabolic cooperation between resident cells of the same species and/or different species is allowed by the biofilm structure. It also exhibits organized internal compartmentalization, which helps the bacterial species in each compartment with different growth requirements. These bacterial cells in a biofilm community may acquire new traits by communicating and exchanging genetic materials[15,16].

It is composed of matrix material consisting of proteins, polysaccharides, nucleic acids, and salt, which makes up 85% by volume, while 15% is made up of cells[17].

An article published by Socransky and Haffajee in 2002[18] compare the development of a biofilm with the development of a city.

Briefly, cities (like biofilms) develop by an initial "attachment" of humans followed by multiplication of the existing inhabitants. They typically spread different directions, forming columnar habitation sites. Cities and biofilms offer their inhabitants many benefits, like shared resources, interrelated activities, protection both from other potential colonizers and from sudden harmful changes in the environment. Individuals in the community can facilitate joint activities and live in a far more stable environment than individuals living in isolation. In cities and biofilms, there are roads, water or sewage pipes. Communication is essential to allow inhabitants to interact optimally. The long-term survival of the human species as well as a species in a biofilm becomes more likely if that species colonizes multiple sites. Thus, detachment of cells from biofilms and establishment in new sites is as important and can establish new colonies[18].

The steps of biofilm development on dental surfaces: The nature of initial bacteria–substrate interaction is determined by physicochemical properties such as surface energy and charge density.

The bacteria adhere to a substrate by bacterial surface structures such as fimbriae, pili, flagella, and extracellular polymeric substances (glycocalyx). Bridges are formed between the bacteria and the conditioning film by these bacterial structures[19].

Molecular-specific interactions between bacterial surface structures and substrate become active. These bridges are a combination of electrostatic attraction, covalent and hydrogen bonding, dipole interaction, and hydrophobic interaction. *Porphyromonas gingivalis, Streptococcus mitis, Streptococcus salivarius, Prevotella intermedia, Prevotella nigrescens, Streptococcus mutans, and Actinomyces naeslundii* are some of the oral bacteria possessing surface structures[15].

With the help of polysaccharide adhesin or ligand formation, which binds to receptors on the substrate, specific bacterial adhesion with a substrate is produced. Microcolony is formed by the monolayer of microbes, which attracts secondary colonizers, and gives rise to the final structure of biofilm. This metabolically active community of microorganisms is a mature biofilm where individuals share duties and benefits. Two types of microbial interactions occur at the cellular level during the formation of biofilm: coadhesion and coaggregation[15].

Some of the reasons why biofilms are such difficult therapeutic targets: Costerton et al. (1999)[20] stated that a wide range of human infections are due to biofilms. These include dental caries, periodontal disease, otitis media, musculoskeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, meloidosis and cystic fibrosis pneumonia[21]. Characteristic of these infections is the persistence and chronicity of the infections as well as the difficulty in their eradication[20].

Generally, multidrug resistance, more than any other property of biofilms, provides a clear demonstration that population behavior is not the sum of the contributions of single cells. Biofilms

are unique multicellular constructions of bacteria from one or several species, in which horizontal genetic transfer may occur easily, thus facilitating crossbreeding of resistance genes[22] and for collateral protection.

In the case of dental biofilms, they are easily accessible and thus allow direct removal and application of antimicrobial agents. However, they are microbiologically very complex[22]. This complexity helps the periodontist in one way and presents problems in another. The complexity helps to assure the therapist that treatment will usually lead to the return of a relatively similar, diverse microbial plaque; hopefully with pathogenic species reduced or eliminated. If treatment virtually eliminated all or most species, the potential for colonization by even more harmful organisms would be very high. On the other hand, the complexity can present difficulties for the therapist. On the other hand, treatments reduce diversity, antibody response controls what can emerge and certain niches are reduced with treatment. The first is knowing which of several potential pathogens in an individual is causing that individual's disease. The second is that the network provided by the community structure may help to “rescue” a suppressed species by providing the essential factors needed for rapid recolonization[23].

## BIOFILM AND ITS ECOLOGICAL RELATIONSHIP

The environmental diversity of the oral cavity promotes the establishment of distinct microbial communities, such as supragingival and subgingival plaque, and tongue coating. The properties of the environment determine which microorganisms can occupy a site, while the metabolic activities of those microbial communities subsequently modify the properties of the environment[24].

Nutrition: Oral microflora uses substrates either derived from the diet of the host or produced by other bacteria. Host substrates are derived from the constituents of saliva and crevicular fluid

(both of which contains low amounts of glucose and proteins). Oral microorganisms are efficient at producing enzymes like proteases, lipases and glycoside hydrolases to degrade and use polymers produced by the host[25]. On example of this interaction is *Veilonella* that can utilize lactic acid produced by *Streptococci* as it cannot ferment carbohydrates on its own. Similarly, certain strains of *Bacteroides melaninogenicus* require Vitamin K produced by other bacteria for growth. Hence there is a symbiotic nutritional relationship between the commensal oral bacteria present in the mouth.

Signaling: Bacteria communicate between themselves by releasing special molecules. This is particularly vital for colony growth and biofilm formation as it sets up the basis of coaggregation between different oral microflora. One example is *Streptococcus gordonii* which produces $H_2O_2$ to kill other oral bacteria, but it does not have catalases to breakdown $H_2O_2$. However, another bacterial species called *Actinnomyces naeslundi* produce catalase, which breaks down some of the $H_2O_2$ that *S. gordonii* cannot hydrolyze[26]. This suggests possible signaling interactions between these two organisms that allow both to flourish together but prevents growth competition with other bacteria.

Adhesion: Biofilms can be formed in a variety of oral cavity surfaces including saliva-coated enamel and dental root surfaces. Primary colonizers do the initial attachment, which results in the formation of microbial monolayers. The adherence is initially a long ranged non-specific reversible interaction, but then followed by an irreversible short ranged adhesion-receptor interaction[27]. With time and arrival of other late colonizers, the microbial monolayer gives rise to a multilayered bio-film as the late colonizers bind to the primary colonizers by co-adhesion. The late colonizers synthesize protein adhesins, which cannot bind to the salivary pellicle but is efficient in binding to

the receptors the primary colonizers. This mechanism where microflora bind to each other to form biofilm is called coaggregation[28]. This is important because the bacteria present in the biofilm community work together to not only outcompete pathogenic bacteria for resources (like space and nutrition) but also have mutualistic nutritional inter-relationships among themselves.

Ecological plaque hypothesis: Historically, the first plaque hypothesis was the "Specific Plaque Hypothesis" in 1976[29]. It proposes that only a few species of the total microflora are actively involved in disease. Secondly, the "Non-Specific Plaque Hypothesis", updated in 1986[30], was the idea that the overall activity of the total microflora could lead to disease was enriched by taking into account difference in virulence among bacteria. Then, a hypothesis was considered that combines key concepts of the earlier two hypotheses: the "Ecological Plaque Hypothesis" by Marsh (1994)[31], which proposes that disease is the result of an imbalance in the microflora by ecological stress resulting in an enrichment of certain disease-related micro-organisms[32].

The key feature is that the enhancement of certain types of bacteria in the oral cavity is directly related to changes in the environment.

Periodontal disease: Health-associated species with an insignificant role in gingival inflammation could significantly contribute to a change in growth conditions favoring pro-inflammatory bacteria. For example, facultative anaerobic *Rothia spp.* has recently been associated with oral health and reduces the oxygen levels in the direct environment[32]. This in turn allows proliferation of strict anaerobes, which include proteolytic Gram-negative bacteria that contribute to triggering the inflammation. Accumulation of commensal microbiota results in an increase in GCF that in turn changes the environment because GCF contains high levels of proteins that are a novel source of nutrients. Furthermore, GCF contains iron that triggers keystone pathogen

mechanisms in *P. gingivalis*. Increased bacterial accumulation triggers more inflammation, which leads to a vicious circle where the host is producing more GCF, more protein and more iron[32].

Environmental changes in periodontitis: The crosstalk between the host and the bacterial biofilm is diverse and bidirectional. The host response and environmental changes induce stress in the biofilm bacteria. For example, the change in the local pH towards an alkaline environment appears to play an important role in the shift towards periodontopathogenic biofilm composition. The biofilm mass is increased in alkaline conditions and, in particular, the intermediate coloniser *F. nucleatum* displays increased adhesion and coaggregation with other bacteria[33]. Another example is the inflammatory environment, the increase of oxidative stress and the inflammatory cytokine IL-1β result in decreased metabolism in periodontal biofilm but still increase various virulence factors as well as biofilm formation. In summary, the environmental changes generated in inflammation favor biofilm formation and appear to drive the bacteria into the shelter provided by the extracellular polymeric substance and the lower metabolic activity.

## MICROBIOLOGY OF PERIODONTAL DISEASES

Historical perspective: The subgingival microbiota is complex and has been recognized as such since the first microscopic examination by Van Leeuwenhoek in 1683[32]. With the development of the light and electron microscopy, cultural techniques and more recently immunologic or DNA probe techniques, researchers were able to understand with more detail what specific microorganisms are present in the biofilm.

In 1975, Listgarten et al.[34] showed that early supragingival plaque has a columnar arrangement of morphologically distinct bacterial species from the tooth surface to the outer surface of the plaque. In addition, in 1974[35] and 1976[36], Listgarten demonstrated that subgingival plaque was

frequently characterized by a zone of gram negative and/or motile species located adjacent to the epithelial lining of the pocket while gram positive rods and cocci appeared to be forming a tightly adherent band of organisms on the enamel or root surface.

Gmur et al. (1989)[37], with probe assessments of plaque, have demonstrated that certain species frequently occur together in subgingival plaque. For example, *P. gingivalis* is almost always observed with *B. forsythus* in the dental biofilm. In addition, Simonson et al. 1992 observed *P. gingivalis* is frequently found with *T. denticola* and Ali et al. (1994)[38] found the same pattern with *F. nucleatun* and *P intermedia* in subgingival plaque samples from deep pockets in a group of adult periodontitis subjects.

Kigure et al. (1995)[39] using immunohistochemical techniques provided graphic demonstration of the relationship between *T. denticola* and *P. gingivalis* in biopsies of subgingival plaque, epithelial and connective tissues from different pocket depths in human periodontitis subjects. Simonson et al. (1992)[40] and Haffajee et al. (1997)[41] demonstrated a reduction in the species of this complex after scaling and root planing.

Technological developments permitted the evaluation of large numbers of bacterial species in large numbers of plaque samples from a wide range of subjects.

In 1988, Socransky et al.[42] observed that some microorganisms are found together in groups that can lead to the development of periodontal diseases. All these previous findings contributed to the development of the study of the microbial complexes in subgingival plaque by Socransky's group.

Using the checkerboard DNA-DNA hybridization technique (developed by Socransky et al. in 1994[43]), Socransky et al. (1998)[2] analyzed subgingival plaque from 185 subjects. Using the

entire database, as well as subsets of data, they presented for the first time a multiple cluster and community ordination analyses. In this study, they proposed a diagrammatic representation (colored coded) of the relationships of species within microbial complexes and between the microbial complexes.

They observed that red complex consists of the tightly related group with *B. forsythus* (now *T. forsythia*) *P. gingivalis* and *T. denticola*. The red complex showed the strongest relationship with the clinical parameters considered most meaningful in periodontal diagnosis. For example, the individual species in the complex as well as the complex itself related very strongly with pocket depth and bleeding on probing. The orange complex is composed by *F. nucleatum, P. intermedia. P nigrescens and P. micros*. This complex is also strongly related with the development of the periodontal diseases and serves as a bridge to the red complex species[2].

The yellow complex consisted of *S. sanguis. S. oralis, S. mitls. S. gordonii* and *S. intermedius*. The green complex comprises three Capnocytophaga species, *C. concisus* and *E. eorrodens*; and the purple complex consisted of *V. parvula* and *A. odontotylicus*. *A. actinotnycetemcomitans, S. taxia* and *A. naeslundii* genospecies were outliers with little relation to each other and the 5 major complexes[2].

In gingivitis, the initial microbial colonization of the plaque biofilm seems to involve members of the yellow, green and purple complexes. They are also sometimes related to the healthy periodontal status. The two species in the purple complex were strongly related to each other and to a lesser extent to members of the orange, green and yellow complexes. The reasons for these relationships among complexes is unclear but Grenier (1996)[44] speculated that it might be that

antagonistic relationships may exist or that environments selective for one group may be less hospitable to a second group of organisms.

New putative pathogens in periodontal disease: The current paradigm of the microbial etiology of periodontitis implicates numerically minor gram-negative anaerobic components of the plaque biofilm, such as *P. gingivalis, T. forsythensis*, and *T. denticola*, as the primary etiologic agents. Although several lines of evidence are available to support an etiologic role for these species, the epidemiologic data linking these species to disease was obtained with closed-ended approaches that would not allow the detection and enumeration of previously unidentified and uncultivated species[45].

In 2001, using cloning and Sanger sequencing, Paster et al.[46] suggested a possible role of cultivable as well as not-yet-cultivable/unrecognized microbial species in the etiology of periodontitis, confirming the idea that the diversity of the oral microbiota was more complex than previously known. Subsequently, a number of other studies using several molecular approaches were published in the periodontal literature[47].

Kumar et al. (2005)[45], using 16S PCR amplification with universal 16S primers of dental plaque samples, followed by cloning and sequencing demonstrated that several genera, many of them uncultivated, were associated with periodontitis, the most numerous of which were gram positive, including *Peptostreptococcus* and *Filifactor*. The genera *Megasphaera* and *Desulfobulbus*, and the levels of several species or phylotypes of *Campylobacter*, *Selenomonas*, *Deferribacteres*, *Dialister*, *Catonella*, *Tannerella*, *Streptococcus*, *Atopobium*, *Eubacterium*, and *Treponema* were elevated in disease. Griffen et al. (2012)[48], not only confirmed these findings, but

also included one Gram-positive bacterium, *Filifactor alocis*, which is potentially important in disease.

Oliveira et al. (2016)[13] demonstrated the association of 5 uncultivated/unrecognized phylotypes *Bacteroidales sp*. HOT 274, *TM7 sp*. HOT 356, *Desulfobulbus sp*. HOT 041, *Fretibacterium sp*. HOT 360, and *Fretibacterium sp*. HOT 362 with generalized chronic and aggressive periodontitis.

This large numbers of additional species will be targets for future research and elucidate major shifts in phylogenetic community composition, these studies could provide the basis for further understanding host–microbe interactions in health and disease[48].

Bacterial analysis in periodontal diseases: Since certain subgingival bacterial species play pivotal roles in the initiation and progression of periodontal diseases, bacterial quantification has been explored as an indicator of disease activity. In addition, several approaches have been proposed for using assessments of subgingival microbiota to guide periodontal therapy. However, because of technical limitations, these studies have focused solely on cultivable species for which polymerase chain reaction (PCR) primers, DNA probes, antibodies, or biochemical assays will be available. Teles et al (2011)[4] have developed RNA-oligonucleotide quantification technique (ROQT) to detect and quantify up to 30 uncultivable/unrecognized and cultivable taxa in 30 biofilm samples. One of the many challenges of investigating the pathogenesis of periodontal diseases is that approximately 50% of the subgingival microbiota are uncultivable/unrecognized.

These “unknown” species may offer important, yet unexplored diagnostic properties. The focus of microbial tests on periodontal pathogens has also been largely biased, showing little attention to beneficial or “bystander” species that might present surprising contributions to

multivariate diagnostic models. Until recently, few tools will be available to study uncultivable/unrecognized species at a site level.

Since the Teles et al. (2011)[4] publication, it became important to improve the technique, to enhance the throughput, sensitivity and specificity of the technique.

## AIM

Thus, the purpose of this study was to optimize the ROQT technique regarding its sensitivity, specificity and cost-effectiveness in terms of laboratory and personal use. Specifically, we propose to analyze the efficacy and sensitivity of the locked nucleic acid (LNA)-modified oligonucleotides probes and to shift to the use of RNA complementary sequences for the preparation of the standards for quantification. In addition, we analyzed the automated extraction of total nucleic acids from clinical samples by using the KingFisher technology (Thermo Fisher Scientific, Waltham, MA). Furthermore, the plan is to use ROQT to quantify selected cultivated and uncultivated bacterial species in subgingival plaque samples. We anticipate that both cultivated and uncultivated species will contribute to the final set of discriminant

# METHODS

## Sample Preparation

Two types of samples were used to test the efficacy, sensitivity, specificity and throughput of the modifications proposed to ROQT protocol: total nucleic acids extracted from a) bacterial cells obtained from reference strains and b) subgingival biofilm samples.

Bacterial cells: as a result of the unavailability of cells from uncultivated/unrecognized bacterial species, cells from reference bacterial species were used as test species for validation purposes in this study (Table 1). The strains were grown in specific media described in Teles et al., 2011[4]. Bacterial cells were harvested from agar plates, placed into 200 µl of RNAse-free TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) and kept at −80°C until extraction of TNA.

Subgingival plaque samples: Subgingival plaque samples were collected from healthy subjects and from patients with severe and moderate periodontitis. Samples were taken separately from mesio-buccal and disto buccal sites using sterile Gracey curettes (HuFriedy, Chicago, IL) and placed in individual 0.5 mL Matrix tubes containing 200 µl RNAse-free TE buffer. Samples were kept at −80°C until TNA extraction. The detailed methods are presented below.

## Extraction of Total Nucleic Acids

Extraction of TNA from all samples was performed using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI) was recommended by the manufacturer and the MagMAX™ Pathogen RNA/DNA Kit-4462359 (Applied Biosystems, Foster City, CA) with

Kingfisher Flex instrument for automated TNA extraction. For the automated protocol, the biofilm samples were added to microcentrifuge tubes containing 450 µL of Lysis/Binding Solution and vortex mixed for 3 minutes. Then, the mixture was centrifuged at 16,000 × g for 2 minutes to clarify the lysate. The sample plate was prepared as follows: 20 µL of prepared Bead Mix was added to one MME-96 Deep Well Plate containing 600 µL of prepared sample (clarified lysate) and 350 µL of 100% isopropanol. The plate was immediately processed in the Kingfisher Flex using the program MagMAX™ provided by the manufacturer. Ninety microliters of purified TNA were obtained in the elution plate and were kept at −80°C until analysis, when the entire sample was laid onto a positively charged nylon membrane.

Ninety microliters of 2% glutaraldehyde and 910 µl 6 × SSC (where 1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) were added to each sample. The final solutions were deposited in individual lanes of a Minislot (Immunetics, Boston, MA), concentrated onto a nylon membrane (Roche-Sigma-Aldrich, St. Louis, MO) by absorption with 15 sheets of 3mm chromatography paper (GE/Whatman $^{TM}$) and fixed onto the membrane by cross-linking using ultraviolet light (UV Crosslinker, Fisher Scientific) and dried at room temperature. The Minislot device permitted the deposition of 30 different plaque samples in individual 'lanes' on a single 15 × 15-cm nylon membrane. In this study, we used 23 samples, 1 quality control sample (QCS) and 6 standards with different concentrations of complementary sequences for quantification of each test species, presented in detail below.

Probe Preparation

Digoxigenin labeled Locked Nucleic Acid (LNA)$^{TM}$ customized oligonucleotides probes were synthesized by Exiqon (Woburn, MA). Probes targeted the 16S ribosomal DNA (rDNA) gene of

4 cultivated species and 19 uncultivated bacterial taxa (Table 2). Sequences were 17-22 nucleotides in length and had minimal secondary structure. The probe panel also included one universal (eubacterial) probe that targeted a conserved region of the bacterial 16S rDNA gene. All probes used in this study were based on sequences previously employed in the Human Oral Microbial Identification Microarray (HOMIM). The full list of probe sequences has been published elsewhere[49].

Hybridization using Oligonucleotide Probes

Before hybridization, the membranes were pre-wetted in 2 × SSC. The membranes were prehybridized in 35 ml of a solution containing 50% formamide, 5 × SSC, 1% casein (Sigma, St Louis MO), 5 × Denhardt's reagent[50], 25 mM sodium phosphate (pH 6.5) and 0.5 mg ml−1 yeast RNA (Roche). Thirty five milliliters of this solution were placed into a plastic hybridization bag containing the membrane. The sealed bag was incubated at 70°C for at least 90 min. Each pre-hybridized membrane with fixed TNA was placed in a Miniblotter 45 (Immunetics, Boston MA), with the "lanes" of TNA at 90 degrees to the channels of the device. A 30 × 45 'checkerboard' pattern was produced.

Probes were diluted in a proprietary hybridization buffer (UltraHyb Oligo buffer; Ambion, Austin, TX). The final concentration of the different probes in each lane in the Miniblotter 45 varied from 2 to 60 pM. The digoxigenin-labeled LNA-oligonucleotide probes, diluted in UltraHyb Oligo buffer, were placed in individual lanes of the Miniblotter. Empty lanes were filled with hybridization solution. The entire apparatus was wrapped in Saran® Wrap and placed in a sealed Ziploc® bag containing 50mL of water to prevent evaporation[43]. Membranes were hybridized at 70°C for 90 min, followed by a high stringency washing (250 mL sterile 2 × SSC,

0.5% SDS at 63°C) in a shaking water bath for 1 hour, replacing the buffer after 30 minutes. Membranes were blocked in a maleic acid buffer (0.1 M maleic acid, 3 M sodium chloride, 0.3 % Tween™ 20, pH 8.0) containing 1% casein on a rotator at room temperature for at least 1 hour.

The membranes were then incubated with a 1:6250 dilution of anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche, Indianapolis, IN, Sigma-Aldrich) on a rotator for 30 min. The membranes were rinsed with maleic acid buffer for 1 min to remove excess antibody and then washed three times with that buffer for 15 min each time. The membranes were equilibrated in 100 ml 'buffer 3' (0.04% magnesium chloride and 2.1% diethanolamine pH 9.5) for 5 min. Finally, 1 ml of a chemiluminescent substrate (CDP Star; Tropix, Bedford, MA) was diluted in 4 ml of 'buffer 3'. The final solution was deposited onto the membrane surface and the membrane was placed in a reaction folder (GE Healthcare-Whatman) The membranes were exposed and the light photons are captured with a GE ImageQuant LAS 4000 for 10 minutes.

Preparation of the Standards for Quantification

The standards for detection using LNA-oligonucleotide probes were a mixture of sequences complementary to the oligonucleotide probes designed to detect both the cultivable and as yet uncultivated taxa. The 'complementary' sequences (Table 2) were synthesized by Sigma (Woodlands, TX). Each membrane contained six standards, each containing 0.004, 0.04, 0.4 or 4 pM of each sequence concentrations for quantification of each test species as well as two standards containing the 'complementary' of the eubacterial probe (4 pm and 40 pm). Ninety microliters 2% glutaraldehyde and 910 µl 6 × SSC were added to the standards and the final solutions of the standards were deposited as the last six lanes of each membrane.

The standard concentrations were based on the estimation that 0.004, 0.04, and 0.4 pM of the complementary probe sequence would approximate $10^5$, $10^6$, and $10^7$ bacterial cells. As bacterial

cells were not available from uncultivated taxa, cultivated bacterial species were employed to test this estimate. Signals were compared from samples containing 10 ng TNA, $10^6$ bacterial cells and 0.04 pM oligonucleotide sequences. These targets were hybridized with probes to the species and the intensities of the signals were compared[4]. One quality control sample was also included in each membrane. The QC sample was comprised of TNA extracted by MagMAX™ Pathogen RNA/DNA Kit on the Kingfisher Flex from 192 subgingival plaque samples collected from subjects with mild to severe periodontitis. The extracted TNA was pooled, aliquoted into microcentrifuge tubes and frozen at – 80 $^0$ C until use.

Determination of Probe Sensitivity and Specificity

Bacterial suspensions were prepared from pure cultures. Upon harvesting, the cell density of each species was adjusted to an optical density at 600 nm of 1 and $10^8$ cells of each species were pipetted into a microcentrifuge tube. The resulting suspension was serially 10-fold diluted and TNA from each dilution was individually extracted using the Masterpure RNA purification kit (Epicentre, Madison, WI). The samples were quantified as described above.

In order to assess the sensitivity of detection of the oligonucleotides, three different probes were compared: conventional oligonucleotides RNA probes, conventional oligonucleotides DNA probes and LNA-oligonucleotides probes. The probes were ran in the membrane against the total nucleic acids extracted from selected cultivable bacterial species used as targets in different concentrations ($10^5$ and $10^6$). The bacteria selected for this experiment were: *T. forsythia, P intermedia, F. nucleatum, P. gingivalis, P. endodontalis, S. anginosus/gordonii, A. geminatus, P. micra* (Fig 1).

To determine the specificity of the probes and the best concentrations of probes to use for all signals to "calibrate" the probe concentrations, two different experiments were performed:

Positive cross-reaction:10 ng TNA from 22 selected bacterial species for the study, as well as 22 LNA-oligonucleotides probes, were laid on the nylon membranes using a Minislot. The membranes were then ‘probed’ in a checkerboard format using all the oligonucleotide probes (Fig 4).

Negative cross-reaction: 10 ng TNA from 96 different bacterial species commonly found in the oral cavity, 22 LNA-oligonucleotides probes, were laid on the nylon membranes using a Minislot. The membranes were then ‘probed’ in a checkerboard format using all the oligonucleotide probes (Fig 5).

Determination of the bead-based kit to extract TNA

In order to optimize the TNA extraction, we tested the automated extraction by using the KingFisher technology (Thermo Fisher Scientific, Waltham, MA). Kingfisher Flex instrument uses magnetic bead technology to automate TNA purification reducing variability and hands-on time utilizing magnetic rods, so we needed to change the extraction kit from Masterpure (scalable salt-precipitation protocol) to a magnetic bead-based extraction kit.

To test what is the best bead-based kit to extract TNA for further use in the automated extraction, 2 kits with different protocols were used and compared with the manual Masterpure protocol. Kit 1: MagMAX™ Pathogen RNA/DNA Kit after cell disruption using the lysing buffer provided by the kit, Kit 2: MagMAX™ Pathogen RNA/DNA Kit after cell disruption using the lysing buffer provided by the kit and using the RNAse step, Kit 3: MagMAX™ Pathogen RNA/DNA Kit after cell disruption using the glass beads protocol (below), Kit 4: Mag-Bind®

Universal Pathogen DNA Kit after cell disruption using the lysing buffer provided by the kit, Kit 5: Mag-Bind® Universal Pathogen DNA Kit after cell disruption using the lysing buffer provided by the kit and using the RNAse step, Kit 6: Mag-Bind® Universal Pathogen DNA Kit after cell disruption using the glass beads protocol (Fig 5). All methods followed the manufactures' protocol.

For the Glass beads lysis, the samples were centrifuge cells at max speed for 10 minutes. The supernatants were discarded and the samples were resuspended in 1ml dilution of lysis buffer (50mM NaH2PO4, 300mM NaCl, 10mM imidazole, pH 8.0) in a 1.5mL tube. Approximately 0.5mL of Glass beads (diameter 500 μm, Thermo Fisher Scientific, Waltham, MA) was added and left on ice for 5 min. The sample were disrupted in a Mini-BeadBeater (Sigma-Aldrich, St. Louis, MO) for 5 min and then centrifuge at 14,000rpm for 20 minutes. The supernatants were transferred to a new tube and the glass beads were discarded.

Pilot Clinical Study

To assess the feasibility of the proposed method, a small pilot study was conducted. Five periodontally healthy individuals and 16 patients with periodontitis were selected for study.

The subjects were recruited for the Biomarkers of Periodontal Disease Progression NIH, NIDCR: 5U01DE021127).

Ethical standards: The investigators ensure that this study was conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research, as drafted by the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in Title 45 of the CFR Part 46 and/or the ICH Guideline for Good Clinical Practice (ICH E6; 62 Federal Register 25691, 1997). The investigators also ensure that the study was conducted

according to the principles of Good Clinical Practice and the Declaration of Helsinki. All aspects of this study was in accordance with all national, state, and local laws of the pertinent regulatory authorities.

Subjects: After signing the consent form, the subject underwent evaluation of eligibility criteria (including periodontal measurements), a medical and dental history, assessment of concomitant medications, urine pregnancy test (in women with childbearing potential), complete oral examination, and full mouth radiographs series (radiographs will not be performed if the subject does not meet all preceding eligibility criteria).

Within 6 weeks after the screening visit, subjects will return for the baseline visit, which marks the beginning of the disease progression and monitoring phase. At this visit, they underwent an interim medical and dental history; complete oral examination; urine pregnancy test (in women with childbearing potential); measurements of height and weight; assessment of concomitant medications; evaluation for unanticipated problems; collection of baseline biological samples; and baseline periodontal measurements.

The periodontal assessments were performed on 6 sites per tooth for all teeth (excluding third molars) and included the presence or absence of gingival redness; presence or absence of plaque, probing depth (PD); measurement of distance from the cemento-enamel junction (CEJ) to the free gingival margin (B measure); clinical attachment level (calculated by subtracting the B measure from the PD); clinical attachment level; presence or absence of BOP; presence or absence of suppuration.

Measurement calibration: Calibration exercises was performed at the Forsyth Institute prior to the start of the study to evaluate intra- and inter-examiner variability. The gold standard was a Dental Hygienist from the School of Dentistry, University of North Carolina at Chapel Hill.

PD and B measure were measured twice, CAL was calculated for each pass, and the median of the measurements was used for analysis. If the difference between the 2 measurements was greater than or equal to 2 mm, the examiner was prompted to measure PD and B measure a third time. When 3 measurements were obtained, the median CAL measurement was used for analysis. When recession is present, B measure was recorded as a negative number, and when the gingival margin is coronal to the CEJ, B measure was recorded as a positive number. Clinical attachment level was calculated by subtracting the B measure from the PD.

Inclusion criteria: To be eligible to participate in this study, a subject must meet all of the following criteria:

Ability to understand, willingness and ability to read and sign the informed consent form. Age of at least 25 years. Ability to understand and follow directions for study procedures. Willingness to comply with all study procedures and be available for the duration of the study. For women with reproductive potential, willingness to use highly effective contraception (e.g., licensed hormonal contraception, intrauterine device, abstinence, or vasectomy in partner). Minimum of 20 natural teeth, excluding third molar teeth; at least 12 of these teeth must be pre-molars, first molars, or second molar.

To ensure a balanced distribution of subjects throughout the spectrum of clinical manifestations, subjects with periodontal disease were stratified according to disease extent and

severity. Thus, there will be 2 sets of inclusion criteria: subjects with mild periodontal loss and subjects with severe periodontal loss.

Subjects with mild periodontal loss must meet all of the following criteria and must not meet the minimum criteria for severe periodontal loss:

At least 4 teeth with at least 1 site of PD of 5 mm or more and concomitant CAL greater than or equal to 2 mm, and radiographic evidence of mesial or distal alveolar bone loss around at least 2 of the affected teeth. Alveolar bone loss is defined as a distance of greater than 2 mm measured radiographically from the CEJ to the crest of the alveolar bone

Subjects with severe periodontal loss must meet all the following criteria:

At least 8 separate teeth with at least 1 site of PD of 5 mm or more and concomitant CAL greater than or equal to 3 mm, and radiographic evidence of mesial or distal alveolar bone loss around at least 2 of the affected teeth. Alveolar bone loss is defined as a distance of greater than 2 mm measured radiographically from the CEJ to the crest of the alveolar bone.

A periodontally healthy subject must meet all of the following criteria:

Any tooth with 3 mm or less PD, irrespective of the attachment level, was acceptable. No teeth with PD of 4 mm or more and concomitant attachment loss, with the exception of the distal of the second molars where a PD of 4 mm and concomitant CAL of up to 2 mm were acceptable. No radiographic evidence of f alveolar bone loss (defined as a distance of greater than 2 mm measured radiographically from the CEJ to the crest of the alveolar bone); with the exception of the mandibular incisors where up to 3 mm of alveolar bone loss measured radiographically from the CEJ to the crest of the alveolar bone will be accepted.

Exclusion criteria: A potential subject will be excluded from participation in this study if he/she meets any of the following criteria:

Presence of orthodontic appliances. Acute necrotizing ulcerative gingivitis or gross tooth decay as determined by the investigator. Root fragments, pericoronitis, endo-perio lesions, or other dental abscesses. Subjects were rescreened after resolution of these dental conditions. Pregnancy or lactation. If a subject meets this criterion, she was rescreened for study participation when she no longer meets the criterion. Requirement for prophylactic antibiotics for dental procedures (e.g., for certain heart and orthopedic conditions). Periodontal or systemic antibiotic therapy in the previous 6 months. If a subject meets this criterion, he/she was rescreened for study participation when he/she no longer met the criterion. Use of cigarettes or other tobacco products within 1 year before the screening visit. If a subject meets this criterion, he/she was rescreened for study participation when he/she no longer met the criterion. Any medical condition that might influence the course of periodontal disease or treatment (e.g., diabetes [irrespective of level of control], human immunodeficiency virus infection or acquired immunodeficiency syndrome, use of medications associated with gingival hyperplasia), chronic use of non-steroidal anti-inflammatory drugs (e.g., for arthritis), defined as the need, or anticipated need, for over 3 weeks of continuous use at the time of enrollment or during the course of the study. The use of low-dose aspirin (≤ 81 mg/day) for prophylaxis was allowed. Current or anticipated use of chronic systemic corticosteroids, cyclosporine, or another systemic immunosuppressive agent. The use of inhaled corticosteroids was allowed. Hypersensitivity to tetracyclines (e.g., tetracycline, doxycycline, minocycline). Participation in a clinical study testing a drug, biologic, device, or other intervention within the last 30 days. If a subject meets this criterion, he/she was rescreened when he/she no longer met the criterion. Any condition or circumstance that, in the opinion of the investigator,

would place the subject at increased risk or preclude his/her full compliance with or completion of the study

The patient will be excluded if the following conditions are noted on oral examination: Oral lichen planus, candidiasis, clinical leukoplakia, clinical erythroplakia, pemphigus, pemphigoid, other recurrent intraoral or perioral vesiculobullous diseases, aphthous ulcerations (major or minor). Subjects presenting with aphthous ulcers were rescreened after 2 weeks. They were eligible if the ulcers have healed and the subject does not have a history of frequent recurrences. Subjects presenting with herpes labialis or intraoral herpes were rescreened after 2 weeks. They were eligible if the lesions have healed and the subject does not have a history of frequent recurrences. If a subject present with a traumatic ulcer, he/she was rescreened in 2-3 weeks. The subject was eligible if the ulcers have healed.

Collection of samples: Subgingival plaque samples were collected at the baseline visit. They were taken using 11/12 sterile Gracey curettes and placed in individual microcentrifuge tubes containing 100 ul RNAse-free TE buffer. Each sample was placed in an individual microcentrifuge tube containing storage buffer and stored at 80º C. Specimen Shipment Samples were stored at the original center of collection. Pre-barcoded vials were used to identify all collected samples, and barcode scanners and software were used to catalogue and track them. Stored samples were shipped in batches to the UNC for analysis on a periodic basis. Samples were kept at −80°C until TNA extraction.

Data analysis

This statistic below will be used to analyze the data from the 4,000 biofilm samples in the "Biomarkers of Periodontal Disease Study" after the RQT technique optimization presented in this thesis.

The images obtained were analyzed using Phoretix Array Software (TotalLab, Newcastle, UK) and the signal intensities of the samples and the standards were measured. Signals were converted to approximate 'counts' by comparison with the standards on each membrane. The 'counts' were computed by estimating that 0.04 pM of target sequences in the standard was equivalent to approximately $10^6$ cells and that 0.004 pM target sequences approximated $10^5$ cells. Absence of signal detection was recorded as zero. In clinical samples, 'counts' for each taxon were averaged within a subject and then across subjects in the periodontitis and periodontally healthy groups, separately. Significance of differences between subject groups was determined using the Mann–Whitney U-test.

## RESULTS

The first step was to assess what type of probe provides more sensitivity, specificity and throughput for the ROQT method. In order to answer these questions, we tested DNA, RNA, and LNA-oligonucleotides probes against their RNA, DNA, TNA, and their poly-T sequences sequences.

We observed that LNA-oligonucleotides probes provide higher sensitivity of detection of the oligonucleotides probes when is compared with conventional oligonucleotides RNA and DNA probes (Fig 1). In addition, there was no difference observed between the RNA and DNA signal and their poly-T sequences (Fig 2). Finally, the sample's reverse RNA provides the strongest signal when compared to the reverse DNA (Fig 3). It is also important to point out that the reverse DNA also provides some weak signal, therefore the decision was to use the total nucleic acid to get both, RNA and DNA, signals.

After the selection of the LNA-oligonucleotides probe, we needed to "calibrate" the probe concentrations in order to optimize the probe and to have a standardize positive control for each oligo sequences from the selected bacteria. After several experiments and concentration adjustments, we found the best concentration of each probe. It can be observed in the figure 4 that each probe reacted against its standard.

Also, in order to assess the specificity of the probes, it was important to know if there was a cross-reaction with the selected probe and other bacterial species commonly found in the oral

cavity. For this experiment, we ran the selected 22 LNA-oligonucleotides probes against 10 ng TNA from 96 different bacteria (Fig 5A). We observed that *P. endodontalis ot273* was the most cross-reacted probe with other bacterial sequences (Fig 5B). Therefore, we decided to exclude this probe from the final membrane.

When different kits and protocols were compared, the best results in terms of sensitivity was observed with the use of the MagMAX™ Pathogen RNA/DNA Kit after cell disruption using the lysing buffer provided by the kit without the RNAse step (Fig 6).

For the final selection of the extraction method, samples from 16 patients with moderate periodontitis were used. 4x4 samples were pulled together and subsequently divide by 4 samples. The TNA were extracted using the manual Masterpure protocol (MP) or the automated protocol using the previously selected kit (MagMAX™ Pathogen RNA/DNA Kit) performed at different stringency conditions (63C and 70C). We observed that the automated method at 63C consistently yields stronger signals in comparison to the manual protocol (Fig 7).

In order to quantify the concentration of bacteria presented in each sample, positive control with 3 different concentration, each containing 0.004, 0.04, 0.4 pM, as well as two standards containing the 'complementary' of the eubacterial probe (0.4 and 4 pm) were added in each membrane. It can be observed in the figure 8 that all the standards could be presented in the curve that could "fit" all samples (not shown).

To assess the reproducibility of the membranes, we decided to add one QC (quality control sample) in each membrane. In order to standardize the sample, 200 biofilm samples from drop-off patients were pulled together and subsequently divided into 200 aliquots. The TNA were extracted and used one sample in each membrane. Figure 9 shows 12 of these samples, we can observe that

they are reproducible. In addition, we have tested different concentration of the elution buffer and glutaraldehyde and we decided to keep the original protocol: 90ul of the elution buffer after the use of 2% glutaraldehyde for the sample preparation.

Finally, we selected biofilm samples from healthy, moderate and severe patients and ran a pilot study to see if different bacteria concentrations could be observed. Figure 10 shows that the most commonly detected uncultivated/unrecognized species in the samples from severe sites were probe *Treponema sp* ot254, *TM7* ot356, *Fretibacterium sp* ot360, Y73 *Selenomonas sp* ot134, 54, L66 and *Desulfobulbus* sp. ot041. In the cultivated segment of the microbiota, the most abundant taxa were *Tannerella forsythia* OT 613, *Oribacterium sp* ot78, K76 *Bacterioidetes sp* ot274, *Fretibacterium fastidiosum* OT 363 and *Porphyromonas gingivalis* ot619.

After improving the sensitivity, specificity of the method we were ready to run all the 4000 samples from the "Biomarkers of Periodontal Disease Progression" study (NIH, NIDCR: 5U01DE021127). The final membrane layout is presented in the figure 11.

## DISCUSSION

Periodontal diseases are polymicrobial infections that can lead to periodontal inflammation and alveolar bone and tooth loss. Periodontal diseases are multifactorial diseases, whose initiation and progression require the participation of several factors, particularly the involvement of subgingival bacteria that contribute to the polymicrobial biofilm formation[51].

Much of our current knowledge of the microbial etiology of periodontitis derives from detailed cultural characterization of the periodontal microbiota[52–56]. Socransky et al., 1998[2] using whole genomic DNA probes and checkerboard DNA–DNA hybridization, characterized periodontal microbial communities classified in distinct complexes that reflected cluster analysis, community ordination and associated disease severity. They identify the 'red complex', a group of three species including *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, that which was strongly associated with each other and with diseased sites. The oral cavity harbors a complex microbiome, with close to 700 predominant taxa[1]. In the past decade, it has become clear that a substantial portion of the oral microbiome was not fully characterized (15%) or could not be cultivated (35%)[1]. Most of the information regarding the association of unrecognized uncultivable/taxa with periodontal health and disease is based on techniques that provide presence/absence data[46,57]. Also, most of those techniques involve sample dilution, sample pooling or PCR amplification of which can add bias to the microbial results[6,8,10,49,58]. The use of next generation sequencing has expanded the breadth of knowledge of the oral microbiota, but only small numbers of subjects and samples have been reported[59,60]. Even though those taxa have

remained undetected in most studies, it is possible that specific uncultivated/unrecognized taxa may play a role in the etiology of oral diseases, or alternatively, they may represent beneficial bacterial species

Quantification is important in the study of periodontal diseases because the differences between periodontal health and disease and before and after therapy are quantitative, rather than qualitative[2,11]. For this reason, quantification of individuals biofilms is necessary. Species quantification in samples from periodontal sites with different clinical status in the same or different oral cavities is a powerful first step in discriminating pathogens from host-compatible species. Our mthod gives us the ability to quantify the number of the bacterial taxa presented in each sample. For this, we need to calculate the concentration of the sample in relation with the known concentration used as a standard (Fig. 8).

Because periodontal diseases are site-specific, can occur in any of the 168 typically evaluated clinical sites and often develops in only a small subset of those sites, there was a need to improve the throughput of microbial techniques, to accommodate the analysis of a larger number of samples per individual[61].

The aim of the present study was to improve a method to detect and quantify uncultivated bacterial species in subgingival biofilm samples in periodontal health and disease. The specificity of the probes used was confirmed by the absence of cross-reactions with any of the 96 bacterial taxa tested, representing the most prominent cultivable oral bacterial taxa. The small clinical study demonstrated the feasibility of the method for its use in clinical trials. The strengths of the proposed method include the absence of pooling, amplification or dilution bias, because an entire individual sample is laid onto the membrane. It allows the quantification of both cultivable and uncultivable bacterial taxa. It is high throughput, in that multiple samples can be analyzed for the levels of

multiple species at the same time on a single membrane and it is relatively inexpensive. The method also has limitations. The standard curve presented has three data points, which enables quantification of taxa in the $10^4$ to $10^7$ cells range in a given sample. However, additional levels of standards can be added to provide a tighter or more comprehensive standard curve. The data presented above were obtained using an image capture by a CCD camera, reaching a dynamic range of 4.8 orders of magnitude and enhancing the accuracy of the method. Although the format of the ROQT may resemble that of the checkerboard DNA–DNA hybridization technique[43,62], it is not meant to represent a 'more sensitive checkerboard'. The techniques differ in the nature of the probes, their target molecules, their hybridization protocols and the nature of the species. The use of ROQT can overcome some of the limitations presented by other molecular biology techniques, including checkerboard DNA-DNA hybridization, real time PCR and 16S rRNA cloning analysis. None of these techniques can quantify the levels of multiple uncultivated species in large numbers of individual biofilm samples simultaneously.

In the present study, the standards for quantification of individual taxa contained 0.004, 0.04 and 0.4 pM of the sequences complementary to the probes. These levels were estimated based on the molecular weight of the 16S rRNA molecule and the number of copies thought to be present in bacterial cells. Subsequent experiments indicated that 0.04 pM yielded signals equivalent to about $0.44 \times 10^5$ bacterial cells. In this study, we compare biofilm samples from patients with moderate periodontitis extracted using the manual Masterpure protocol (MP) and the automated protocol (MagMAX™ Pathogen RNA/DNA Kit with Kingfisher Flex instrument) performed at different stringency conditions (63C and 70C). It can be observed that the automated method consistently yields stronger signals in comparison to the MP protocol (Figure 7). The possibility of use an automated TNA extraction allows an optimization of entire process.

ROQT hybridization technique offers a number of advantages. The principal advantage is that large numbers of DNA, RNA, tissue, bacterial or viral samples could be tested with multiple probes at the same time[62]. ROQT represents a hybrid of checkboard and HOMIM: combines the ability to assess levels of taxa, without an amplification step (aliquot and PCR can introduce bias, the entire sample is used); have the ability to detect uncultivated/unrecognized taxa thanks to the specificity of the oligonucleotide probe that are the same ones used in HOMIM[46,57]. One major difference and an advantage in relation to both techniques is that it targets RNA, rather than DNA. DNA based techniques do not differentiate live/dead cells, since DNA can be present even after cell death. In contrast, because rRNA is essential to basic cellular metabolism and it is thought to degrade soon after cell death, RNA targeting techniques provide better representation taxa actively involved in health and disease. ROQT can quantify the levels of multiple uncultivated species in large numbers of individual biofilm samples simultaneously and is a high-throughput method for bacterial enumeration in clinical biofilm samples and have been used in several studies[12,13,47,51].

The short length and high sequence similarity between closely related microRNAs makes it hard to detect them with sufficient specificity and sensitivity. Locked nucleic acid (LNA) is one type of nucleotide analogues having a methylene bridge between O2′ and C4′ atoms of ribose to form a bicyclic ribosyl structure. It is the bridged structure that 'locks' effectively the ribose in the C3′-endo sugar pucker conformation, which is observed primarily in A-form DNA and RNA[63]. LNA modified probes can enhance the binding affinity towards their complementary DNA strands due to the improved base stacking and phosphate backbone preorganization[64], and can improve their capability of discriminating the mismatched base pairs[65]. For this reason, the use of LNA-oligonucleotides probes is superior to DNA technologies, because only the perfect match with the complementary RNA will occur. For comparison, we tested DNA oligonucleotides probes, RNA

oligonucleotides probes and LNA oligonucleotides probes (Figure 1-3). Results showed that DNA and RNA probes exhibited lower signal intensity than LNA-modified probes under the same

In the cultivated segment of the microbiota, we observed that the most abundant taxa were *Tannerella forsythia* ot613, *Fretibacterium fastidiosum* ot363, L66 *Oribacterium sp* ot78, K76 *Bacterioidetes sp* ot274*,* and *Porphyromonas gingivalis* ot619**.** Several studies showed that these bacteria were significantly more abundant in the periodontal patients with more inflammation[2,77]. For example, Oliveira et al. (2016)[13] observed that subgingival biofilm from patients either with Aggressive and Chronic Periodontitis harbors higher amounts of the cultivated bacteria F. *fastidiosum* ot363, *T. forsythia* ot613 and *P. gingivalis* ot619, and the uncultivated *Fretibacterium sp* ot360, Y73 *Selenomonas sp* ot134, and *TM7* ot356 when compared to the healthy sites. In addition, recent studies using 454 pyrosequencing to characterize healthy and periodontitis microbial communities[48,78], the overall picture of bacterial associations with health and disease agree with the initial descriptions of the different oral microbial complexes described by Socransky et al. (1998)[2].

Due to the complexity of the oral community the initial causes for transition from a healthy microbial community to a dysbiotic one is still not known. It is important identify the presence and the levels of taxa involved in the dysbiotic environment that precedes the disease initiation. ROQT can examine large numbers of biofilm samples from large numbers of subjects helping in identify the more relevant uncultivated and unrecognized taxa. Identify these taxa is very important to further cultivation and identification of pathogenic mechanism involved in the establishment and prevention of periodontal disease.

## CONCLUSION

The use of automated TNA extraction, LNA probes and RNA standards enhances the sensitivity, specificity, throughput, and cost and time-effectiveness of ROQT.

# TABLES

Table 1.

| Candidate Probes | In NT list? | Evidence (0-6) |
|---|---|---|
| Porphyromonas gingivalis | n/a | 4^ |
| Tannerella forsythia | n/a | 3^ |
| Filifactor alocis | n/a | 6 |
| Porphyromonas endodontalis | n/a | 4 |
| Fretibacterium fastidiosum | n/a | 4 |
| **Desulfobulbus sp. oral taxon 041** | y | 3 |
| **Fretibacterium (Synergistetes) sp. oral taxon 360** | y | 3 |
| ***Selenomonas sp. oral taxon 134_442**** | y | 0 |
| **Tannerella sp. oral taxon 286** | y | 1 |
| TM7 sp. oral taxon 356 | n | 1 |
| ***TM7[G-1] sp. oral taxon 346_349**** | y | 1 |
| ***Treponema sp. oral taxon 254_256_508_517**** | y | 0 |
| **Actinomyces sp. oral taxon 170** | y | 3 |
| **Actinomyces sp. oral taxon 175** | y | 3 |
| **Bacteroidales[G-2] sp. oral taxon 274** | y | 3 |
| **Clostridiales[F-2][G-1] sp. oral taxon 075** | y | 1 |
| **Megasphaera sp. oral taxon 123** (Peptostreptococcacea sp. OT 123) | y | 1 |
| **Oribacterium sp. oral taxon 078** | y | 1 |
| ***Peptostreptococcaceae[11][G-4] sp. oral taxon 103_369**** | y | 2 |
| Porphyromonas sp.OT 279 | n | 3 |
| Prevotella sp. OT 306 | n | 2 |
| **SR1[G-1] sp. oral taxon 345** | y | 1 |
| ***Streptococcus sp. oral taxon 070_071**** | y | 1 |

Table 1. List of the bacteria selected for this study, their presence in the NT list and the evidence of their role in periodontal disease. NT: New Technologies. The evidence number is the of times it was mentioned in literature. Uncultivated bacteria are in bold letters.

Table 2.

| Human Oral Taxon (HOT) probe target | Status | Probe_sequence 5'-3' | Reverse Complement RNA Sequence | Reverse Complement DNA Sequence | Previous name |
|---|---|---|---|---|---|
| *Actinomyces sp. HOT 170* | Unnamed - Cultured | ACACCCACCACAAAGGA | UCCUUUGUGGGUGGGUGU | TCCTTTGTGGTGGGTGT | |
| *Actinomyces sp. HOT 175* | Unnamed - Cultured | AGCAAAAACCGGTCCCTT | AAGGGACCGGUUUUUGCU | AAGGGACCGGTTTTTGCT | |
| *Bacteroidetes[G-2] sp. HOT 274* | Unnamed - Cultured | AGATGCCTCTTCCGTTTAC | GUAAACGGAAGAGGCAUCU | GTAAACGGAAGAGGCATCT | |
| *Oribacterium sp. HOT 78* | Unnamed - Cultured | AGGGAAAAGGCATTACGC | GCGUAAUGCCUUUUCCCU | GCGTAATGCCTTTTCCCT | |
| *Porphyromonas pasteri HOT 279* | Recently Cultivated and Named | CTTGGAGTAGGATGCCC | GGGCAUCCUACUCCAAG | GGGCATCCTACTCCAAG | Porphyromonas sp. HOT 279 |
| *Prevotella sp. HOT 306* | Unnamed - Cultured | CCCAAGCTTAACCTGATG | CAUCAGGUUAAGCUUGGG | CATCAGGTTAAGCTTGGG | |
| *Streptococcus sp. HOT 070* | Unnamed - Cultured | ATGCGATAATCCATTTTATGCG | CGCAUAAAAUGGAUUAUCGCAU | CGCATAAAATGGATTATCGCAT | |
| *Tannerella sp. HOT 286* | Unnamed - Cultured | TCTGTTGTAGGTAGGTTGC | GCAACCUACCUACAACAGA | GCAACCTACCTACAACAGA | |
| *Desulfobulbus sp. HOT 041* | Phylotype - Uncultured | TGTTATTCGCTGCCTTGCA | UGCAAGGCAGCGAAUAACA | TGCAAGGCAGCGAATAACA | |
| *Fretibacterium sp. HOT 360, 362* | Phylotype - Uncultured | GCAGCGTCGTCAATGTTT | AAACAUUGACGACGCUGC | AAACATTGACGACGCTGC | |
| *Megasphaera sp. HOT 123* | Phylotype - Uncultured | TCTACGCCCTTCACTCAA | UUGAGUGAAGGGCGUAGA | TTGAGTGAAGGGCGTAGA | |
| *Peptostreptococcaceae(11)[G-4] HOT 103, 369* | Phylotype - Uncultured | ACTCCCAACTTTACTCGG | CCGAGUAAAGUUGGGAGU | CCGAGTAAAGTTGGGAGT | |
| *Ruminococcaceae [G-1] sp. HOT 075* | Phylotype - Uncultured | AGGGATTTCACATACGACTT | AAGUCGUAUGUGAAAUCCCU | AAGTCGTATGTGAAATCCCT | Clostridiales[F-2][G-1] sp. HOT 75 |
| *Selenomonas sp. HOT 134; 442* | Phylotype - Uncultured | AACCCGGTTTTCGTCCC | GGGACGAAAACCGGGUU | GGGACGAAAACCGGGTT | |
| *SR1[G-1] sp _HOT 345* | Phylotype - Uncultured | GTCATTCGTCTTCTGCCA | UGGCAGAAGACGAAUGAC | TGGCAGAAGACGAATGAC | |
| *TM7 sp. HOT 356* | Phylotype - Uncultured | CGAACAACAAGCTATCGG | CCGAUAGCUUGUUGUUCG | CCGATAGCTTGTTGTTCG | |
| *Treponema sp. HOT 254, 256, 508, 517* | Phylotype - Uncultured | CTGAGTCTTACCCCAACA | UGUUGGGGUAAGACUCAG | TGTTGGGGTAAGACTCAG | |
| *Porphyromonas gingivalis HOT 619* | Named species-Pathogen | AGTCGCAGTATGGCAAG | CUUGCCAUACUGCGACU | CTTGCCATACTGCGACT | |
| *Tannerella forsythia HOT 613* | Named species-Pathogen | TTGCGGGCAGGTTACATA | UAUGUAACCUGCCCGCAA | TATGTAACCTGCCCGCAA | |
| *Filifactor alocis HOT 539* | Named species-Proposed Pathogen | CTTCTATCATCTTCGCCC | GGGCGAAGAUGAUAGAAG | GGGCGAAGATGATAGAAG | |
| *Fretibacterium fastidiosum HOT 363* | Recently Cultivated and Named-Proposed Pathogen | GTGTTACCACTTCACGAC | GUCGUGAAGUGGUAACAC | GTCGTGAAGTGGTAACAC | |
| Universal 16S Probe | Eubacterial | CTGCTGCCTCCCGTAGG | CCUACGGGAGGCAGCAG | CCTACGGGAGGCAGCAG | |

HOT, human oral taxon. Oral taxon designations for uncultivated/unrecognized taxa are provided in accordance with the Human Oral Microbiome Database (www.homd.org), when available; GenBank accession numbers can also be found in the website.

Table 2. List of the bacteria selected for this study with the respective probe sequence.

## FIGURES

Figure 1.

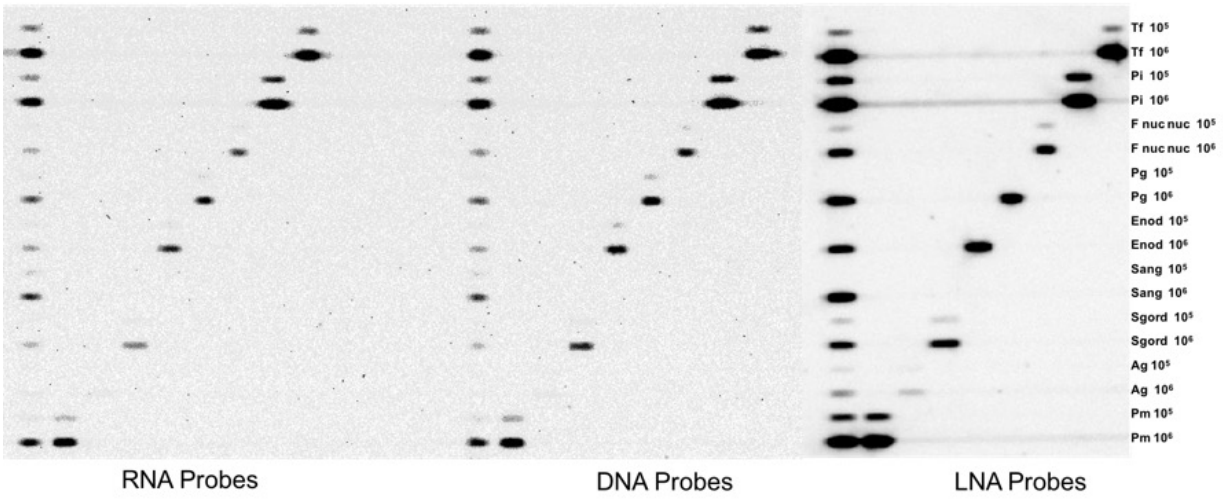


Figure 1. Comparison between conventional oligonucleotides RNA probes, conventional oligonucleotides DNA probes and LNA-oligonucleotides probes. Total nucleic acids extracted from selected cultivable bacterial species were used as targets in the horizontal lanes, cell targets were used in concentrations $10^5$ and $10^6$ respectively. The left-most lane is a universal probe to assess the amount of TNA on the membrane.

Figure 2.

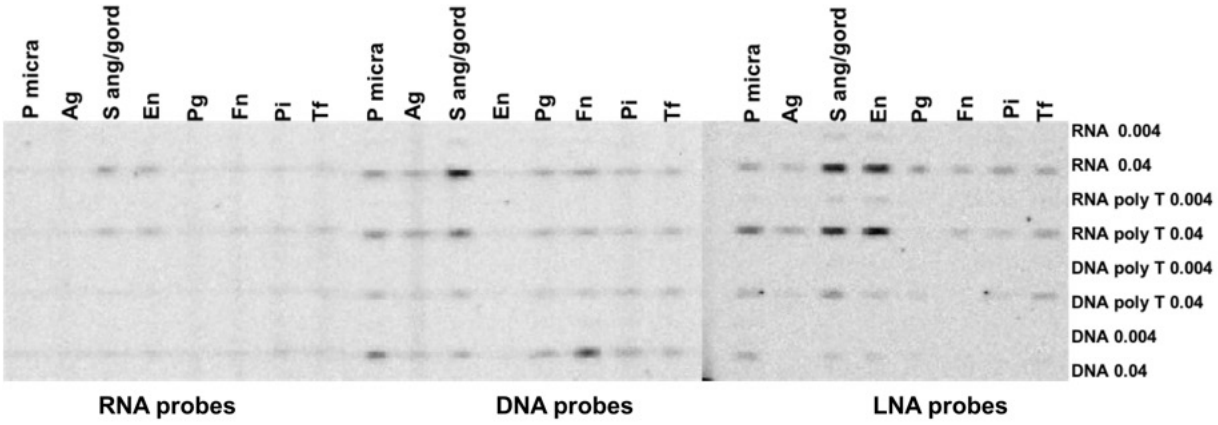


Figure 2. Membrane with different types of oligonucleotide targets in pmol against the cultivable probes.

Figure 3.

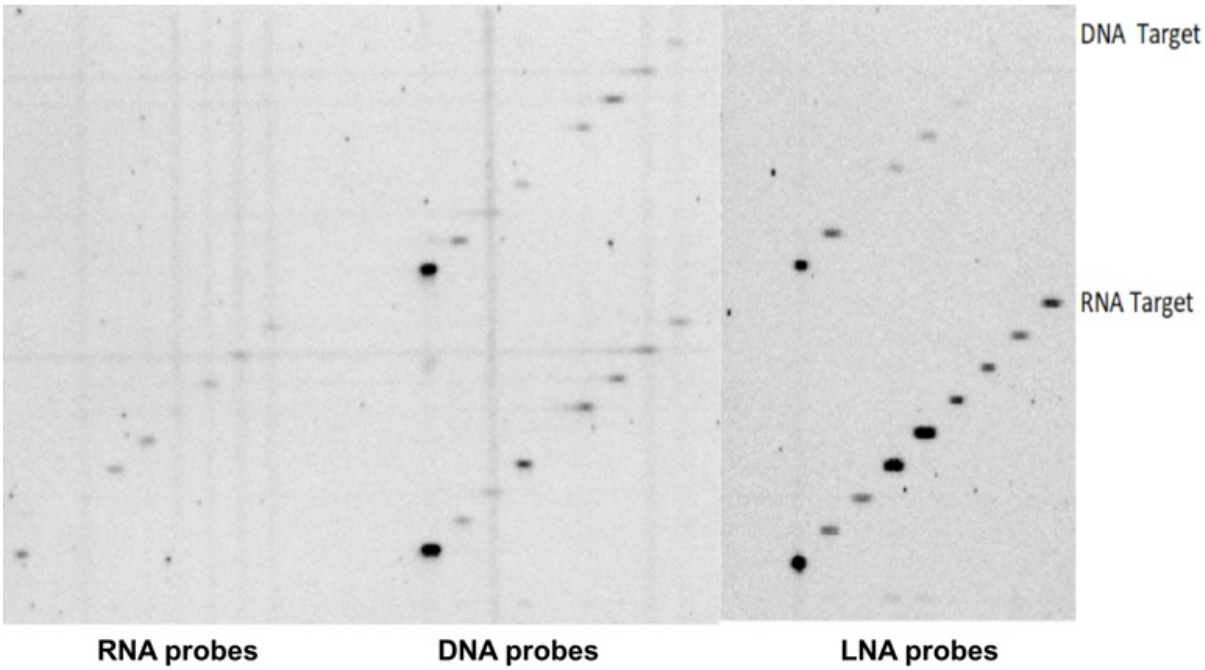


Figure 3. RNA oligonucleotide quantification technique (ROQT) membrane used to assess the different oligonucleotides DNA, RNA and LNA probes. Reverse DNA and RNA 0.04pmol from selected bacterial species were used as targets in the horizontal lanes. Oligonucleotides DNA, RNA and LNA probes were hybridized against these targets.

Figure 4.

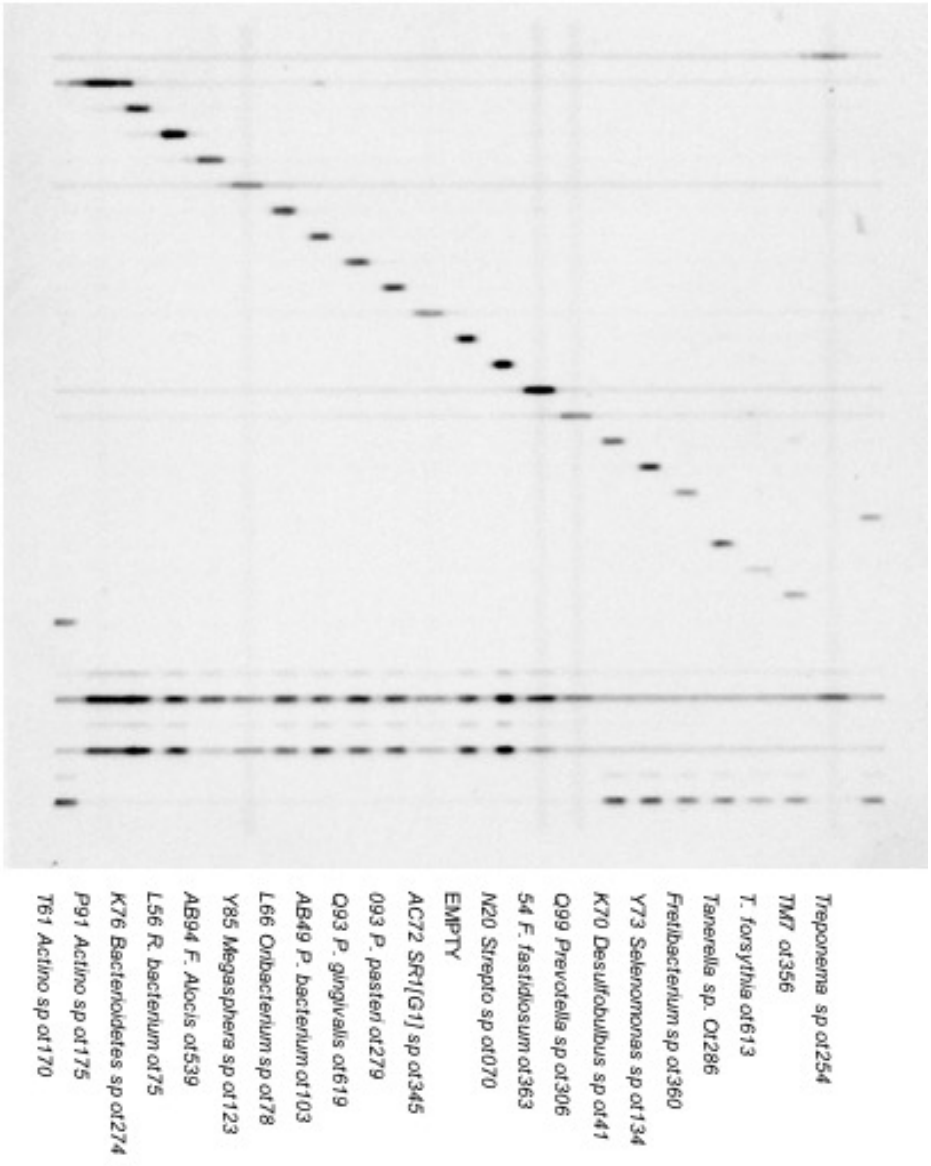


Figure 4. The specificity of the probes was tested using 10ng of RNA oligo sequences from each of the selected bacteria. Because of the unavailability of cells from uncultivated taxa, probes from those species were tested against their complementary oligonucleotide sequences. This membrane was used to determine the best concentrations of probes to use for all signals to be the same intensity so as to "calibrate" the probe concentrations.

Figure 5.

A

Table 1: Reference bacterial strains used to test specificity of oligonucleotide probes.

| | |
|---|---|
| *Actinomyces georgiae* (49285) | *Lactobacillus fermentum* (14931) |
| *Actinomyces gerencseriae* (23860) | *Lactobacillus oris* (49062) |
| *Actinomyces israelii* (12102) | *Lactobacillus salivarius* (11741) |
| *Actinomyces meyeri* (35568) | *Legionella pneumophila* (33153) |
| *Actinomyces naeslundii I* (12104) | *Leptotrichia buccalis* (14201) |
| *Actinomyces naeslundii I* (43146) | *Moraxella catarrhalis* (24240) |
| *Actinomyces odontolyticus* (17929) | *Neisseria mucosa* (19696) |
| *Aggregatibacter actinomycetemcomitans1* | *Olsenella uli* (49627) |
| *Atopobium rimae* (49626) | *Parvimonas micra* (33270) |
| *Bacteroides fragilis* (25285) | *Peptostreptococcus anaerobius* (27337) |
| *Bifidobacterium dentium* (27534) | *Porphyromonas endodontalis* (35406) |
| *Campylobacter concisus* (33237) | *Porphyromonas gingivalis* (33277) |
| *Campylobacter curvus* (35224) | *Prevotella dentisola* (35308) |
| *Campylobacter gracilis* (33236) | *Prevotella intermedia* (25611) |
| *Campylobacter rectus* (33238) | *Prevotella loeschii* (15930) |
| *Campylobacter showae* (51146) | *Prevotella melaninogenica* (25845) |
| *Campylobacter sputorum ss sputorum* (35980) | *Prevotella nigrescens* (33563) |
| *Campylobacter ureolyticus* (33387) | *Prevotella oris* (33573) |
| *Capnocytophaga gingivalis* (33624) | *Prevotella tannerae* (51259) |
| *Capnocytophaga ochracea* (33596) | *Propionibacterium acnes I* |
| *Capnocytophaga sputigena* (33612) | *Propionibacterium acnes II* |
| *Dialister invisus* (G8427) | *Propionibacterium propionicum* (34157) |
| *Eikenella corrodens* (23834) | *Rothia dentocariosa* (17931) |
| *Enterobacter sakazakii* (12868) | *Selenomonas artemidis* (43528) |
| *Enterococcus aerogenes* (13048) | *Selenomonas infelix* (43523) |
| *Enterococcus faecalis* (10100 & 29212) | *Selenomonas noxia* (43541) |
| *Escherichia coli* (10798) | *Selenomonas sputigena* (35185) |
| *Eubacterium brachy* (33089) | *Slackia exigua* (700122) |
| *Eubacterium limosum* (8486) | *Staphylococcus aureus* (33591) |
| *Eubacterium nodatum* (33099) | *Staphylococcus epidermidis* (14990) |
| *Eubacterium saburreum* (33271) | *Staphylococcus warneri* (27836) |
| *Eubacterium saphenum* (49989) | *Streptococcus anginosus* (33397) |
| *Filifactor alocis* (35896) | *Streptococcus constellatus* (27823) |
| *Fusobacterium naviforme* (25832) | *Streptococcus gordonii* (10558) |
| *Fusobacterium necrophorum* (25286) | *Streptococcus intermedius* (27335) |
| *Fusobacterium nucleatum ss. nucleatum* (25586) | *Streptococcus mitis* (49456) |
| *Fusobacterium nucleatum ss. polymorphum* (10953) | *Streptococcus mutans* (25175) |
| *Fusobacterium nucleatum ss. vincentii* (49256) | *Streptococcus oralis* (35037) |
| *Fusobacterium periodonticum* (33693) | *Streptococcus parasanguins* (15912) |
| *Gemella haemolysans* (10579) | *Streptococcus pneumoniae* (49619) |
| *Gemella morbillorum* (27824) | *Streptococcus salivarius* (27941) |
| *Granulicatella adiacens* (49175) | *Streptococcus sanguinis* (10556) |
| *Haemophilus aphrophilus* (33389) | *Streptococcus vestibularis* (49124) |
| *Haemophilus influenza* (33533) | *Tannerella forsythia* (43037) |
| *Haemophilus paraphrophilus* (29242) | *Treponema denticola* (B1) |
| *Haemophilus segnis* (33393) | *Veillonella atypica* (17744) |
| *Lactobacillus acidophilus* (4356) | *Veillonella dispar* (17748) |
| *Lactobacillus brevis* (14869) | *Veillonella parvula* (1079 |

All cultivated strains were obtained from the American Type Culture Collection (ATCC number in parenthesis), except for Treponema denticola (B1), which was obtained from The Forsyth Institute.

1: ATCC strains 43718 and 29523

2: ATCC strains 51827 and 51828

B

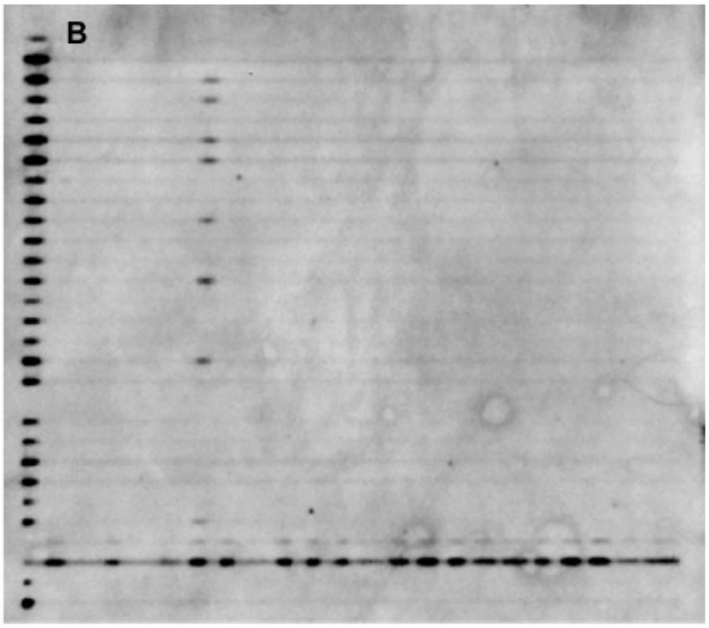

Figure 5. The specificity of the probes was tested using 10 ng of TNA from bacteria not selected for the study to see their cross-reaction. Panel A: List of the bacteria selected to analyze if there is a cross-reaction between these bacteria the ones selected for the study. Panel B: membrane showing some cross-reactions.

Figure 6.

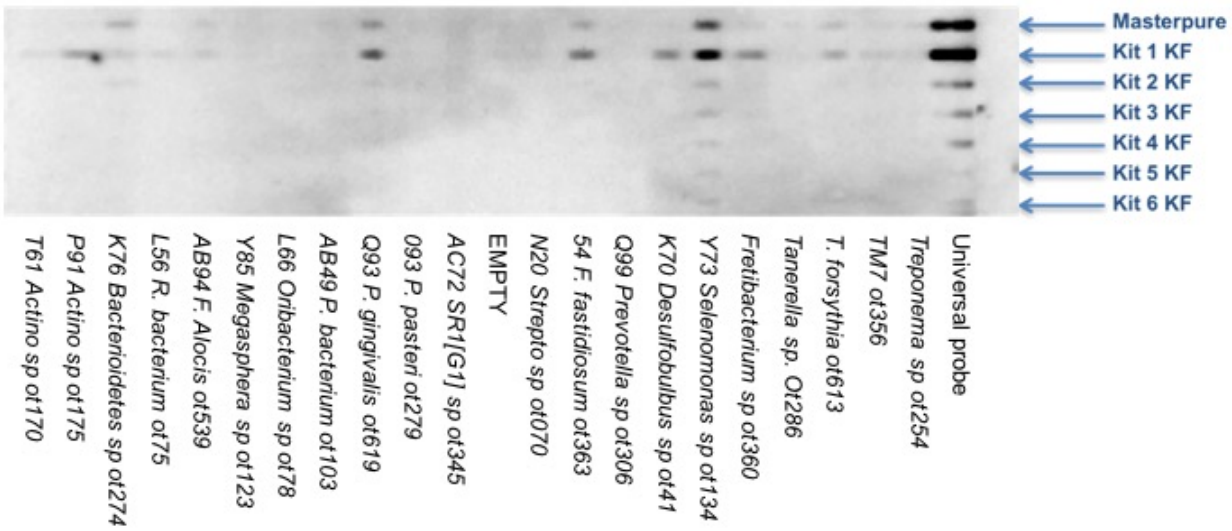


Figure 6. RNA oligonucleotide quantification technique (ROQT) membrane after TNA extraction using different magnetic based kit compared against the standard kit Materpure. Kit 1: MagMAX™ Pathogen RNA/DNA Kit after cell disruption the lysing buffer provided by the kit, Kit 2: MagMAX™ Pathogen RNA/DNA Kit after cell disruption the lysing buffer provided by the kit and using the RNAse step, Kit 3: MagMAX™ Pathogen RNA/DNA Kit after cell disruption with glass beads, Kit 4: Mag-Bind® Universal Pathogen DNA Kit after cell disruption the lysing buffer provided by the kit, Kit 5: Mag-Bind® Universal Pathogen DNA Kit after cell disruption the lysing buffer provided by the kit and using the RNAse step, Kit 6: Mag-Bind® Universal Pathogen DNA Kit after cell disruption with glass beads.

Figure 7.

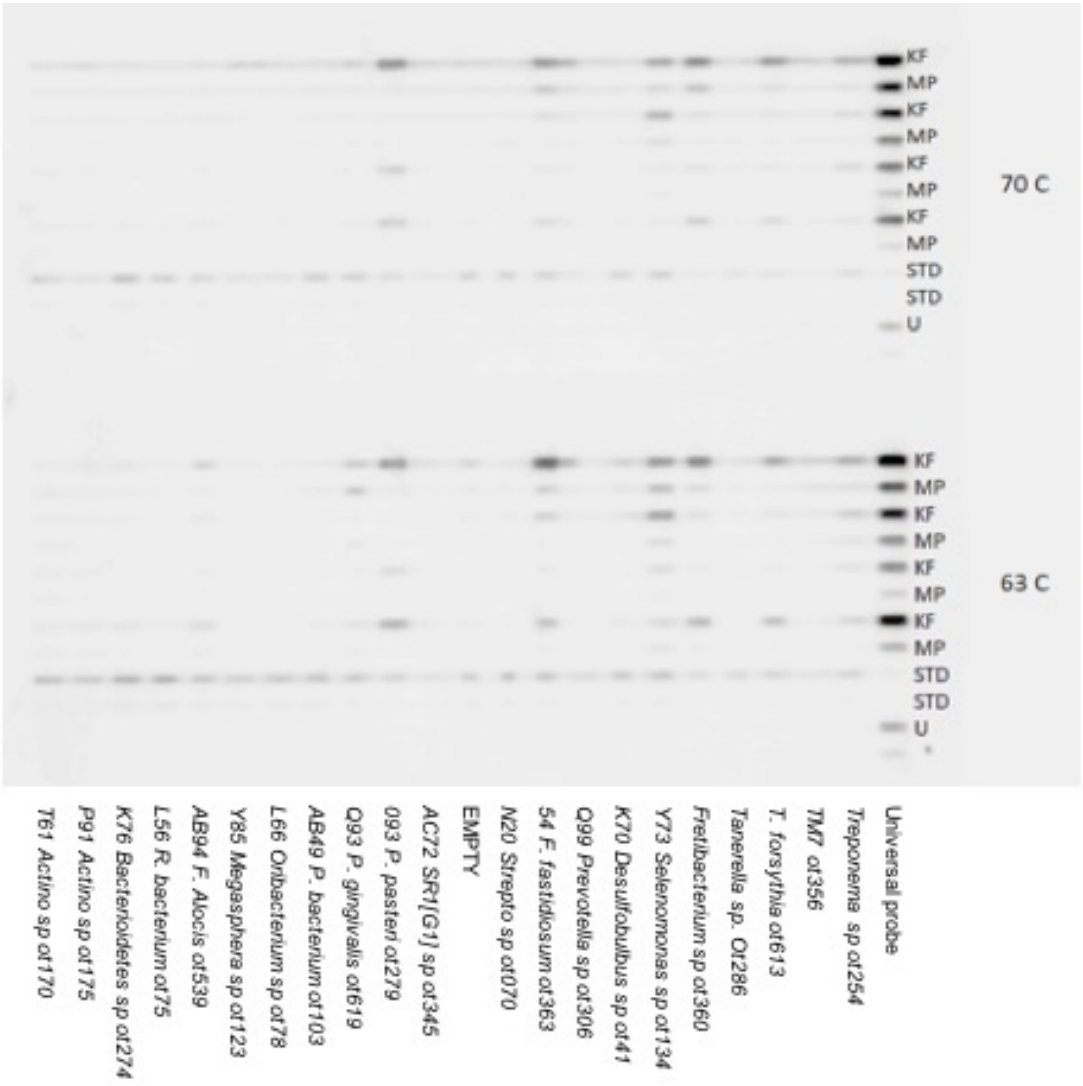


Figure 7. Figure shows samples extracted using the manual Masterpure protocol (MP) and the automated protocol (MagMAX™ Pathogen RNA/DNA Kit with Kingfisher Flex instrument) performed at different stringency conditions (63◦C and 70◦C). Samples represent 4 patients with severe periodontal disease. The sample of each patient was divided in two and the TNA extracted with MP and MagMAX™ Pathogen RNA/DNA Kit and were hybridized with 23 probes (including the Universal probe).

Figure 8.

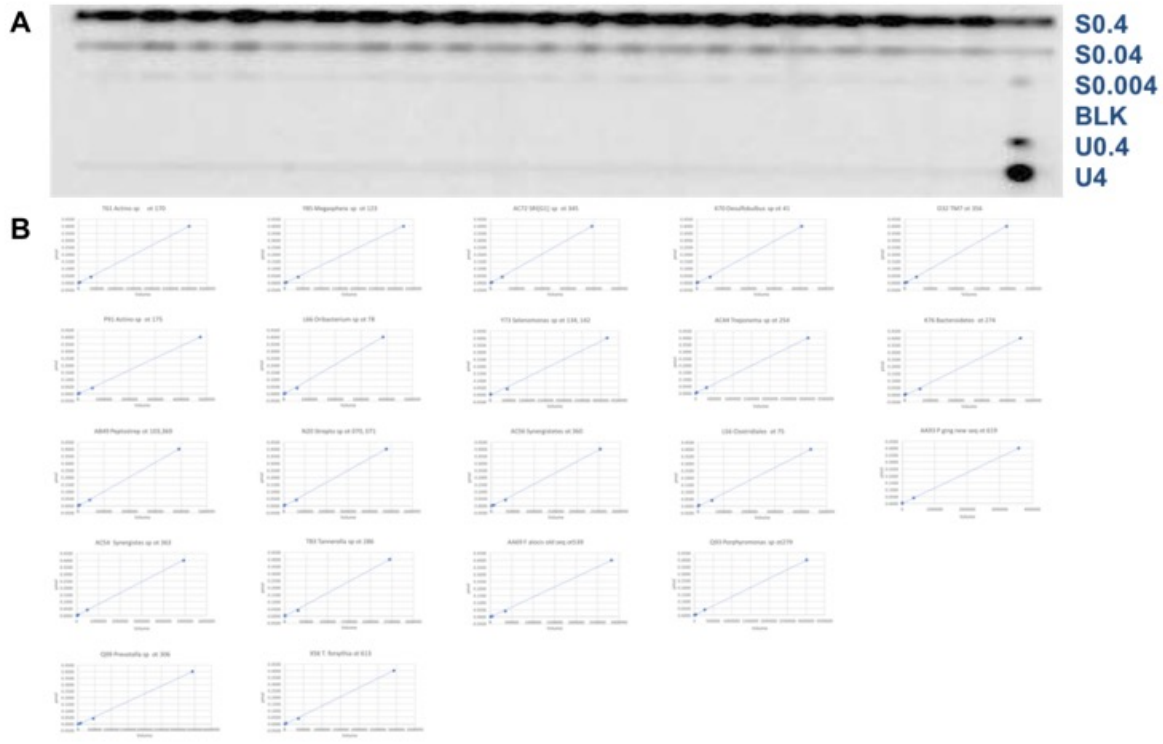


Figure 8. Panel A: RNA oligonucleotide quantification technique (ROQT) membrane showing the standards and the universal probe. Each membrane contained three standards, each containing 0.004, 0.04, 0.4 pM of each sequence concentrations for quantification of each test species as well as two standards containing the 'complementary' of the eubacterial probe (0.4 and 4 pm). Panel B: Graphic curve using the band intensity analyzed in the ROQT membrane.

Figure 9.

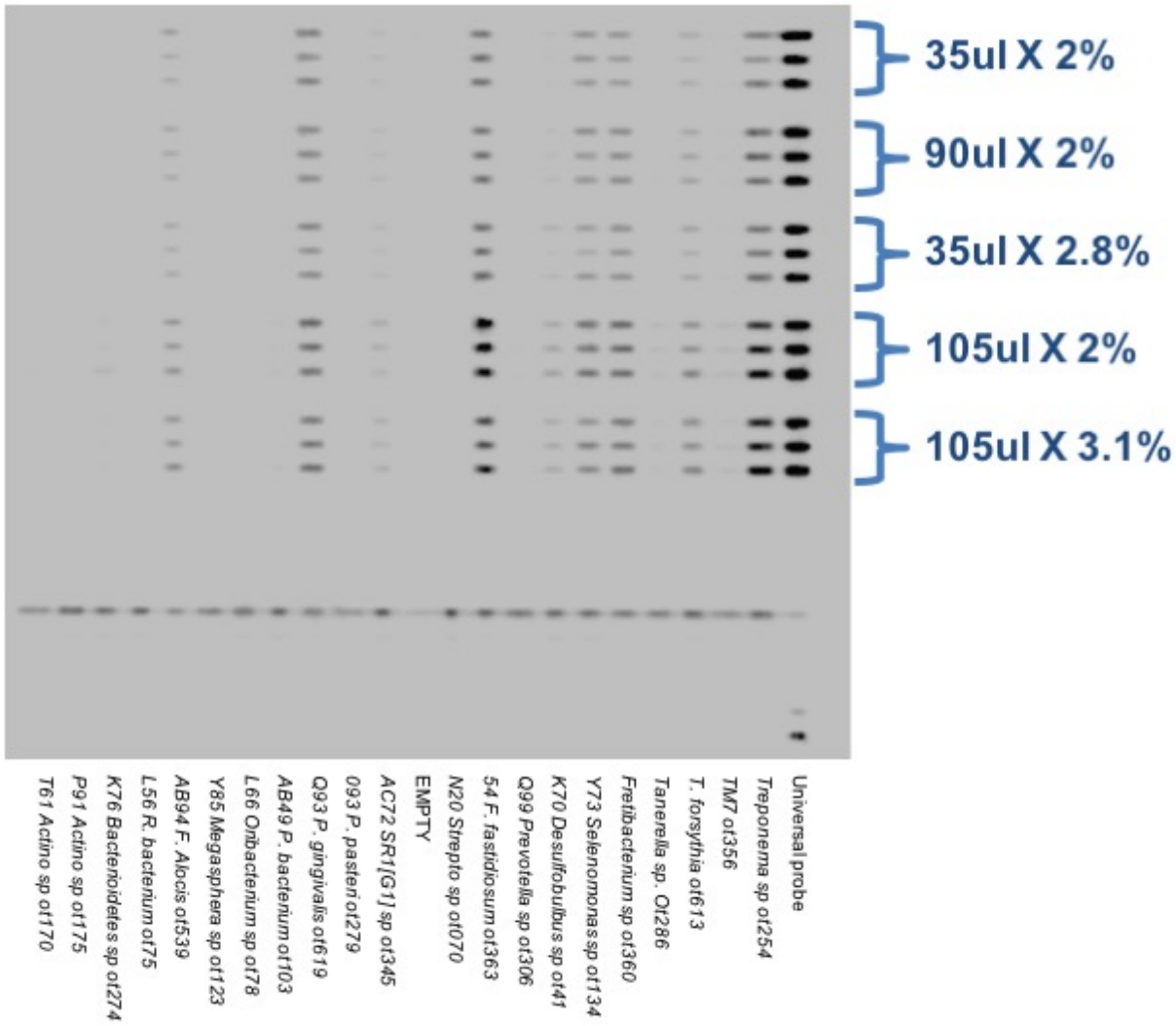


Figure 9. Figure shows different concentration of the elution buffer and glutaraldehyde. 200 TNA samples were pooled together and subsequently divided in 200 samples to serve as a quality control (QC) sample.

Figure 10.

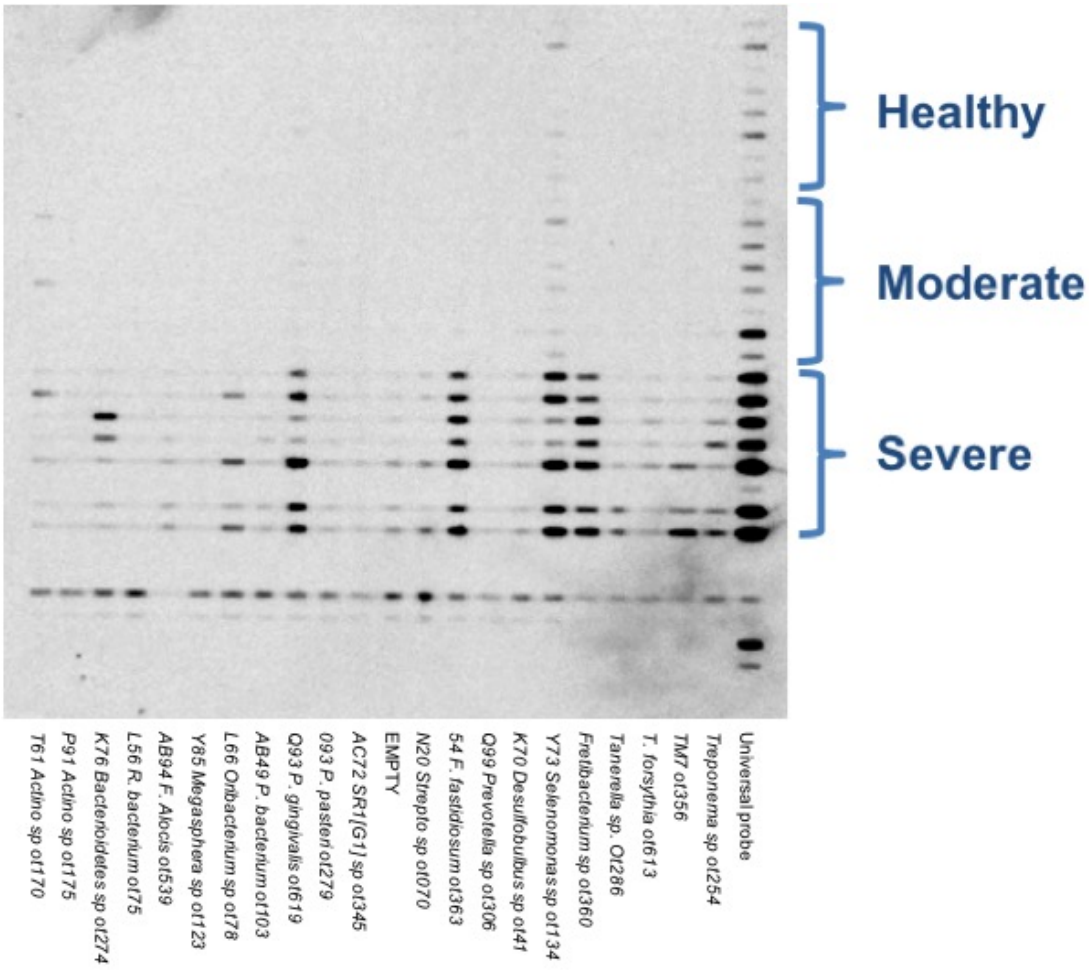


Figure 10. A checkerboard membrane showing hybridization of clinical samples with LNA_oligonucleotide probes. Probes for cultivated species and as yet uncultivated species are listed across the bottom. Each horizontal lane represents the total nucleic acids (TNA) from a sample from Health, Moderate, and Severe periodontal disease sites. Each membrane contained standards (0.004, 0.04, 0.4 pM) and the standards containing the 'complementary' of the eubacterial probe (0.4 and 4 pm).

Figure 11.

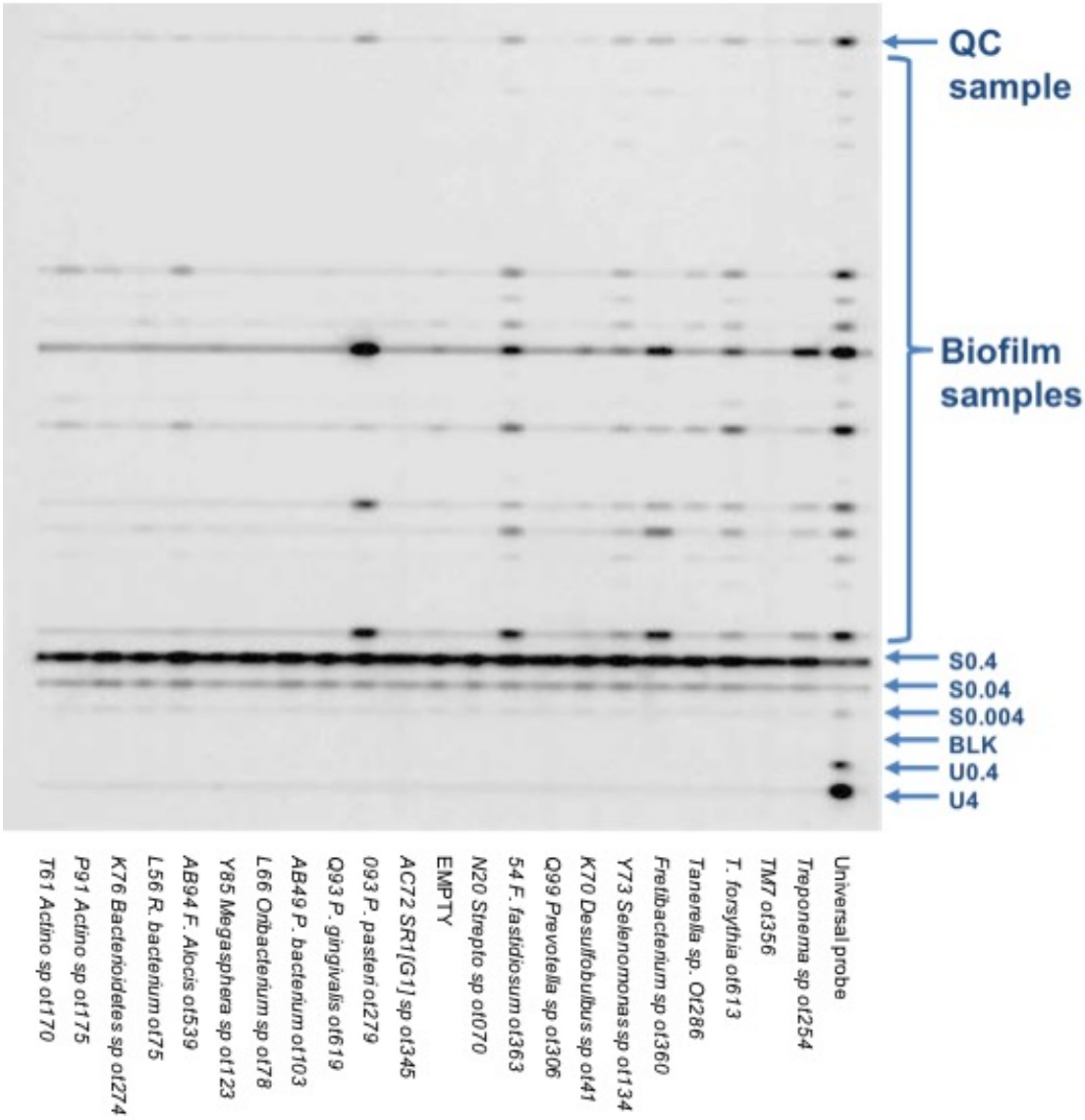


Figure 11. A final checkerboard membrane showing hybridization of clinical samples with LNA_oligonucleotide probes. Probes for cultivated species and as yet uncultivated species are listed across the bottom. Each horizontal lane represents the total nucleic acids (TNA) from biofilm sample. Standards comprised a mixture of 'complementary' sequences from all the test taxa at 0.4, 0.04 and 0.004 pM, and the Universal probes respectively. (U standards are 0.04 with the S0.004 then 0.4 and 4.0).

## REFERENCES


1. Dewhirst, F. E. *et al.* The human oral microbiome. *J. Bacteriol.* **192,** 5002–5017 (2010).
2. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* **25,** 134–144 (1998).
3. Chen, T. *et al.* The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* **2010,** baq013-baq013 (2010).
4. Teles, F. R. F. *et al.* RNA-oligonucleotide quantification technique (ROQT) for the enumeration of uncultivated bacterial species in subgingival biofilms. *Mol. Oral Microbiol.* **26,** 127–139 (2011).
5. Teles, F. R., Haffajee, A. D. & Socransky, S. S. The reproducibility of curet sampling of subgingival biofilms. *J. Periodontol.* **79,** 705–13 (2008).
6. Amann, R. & Ludwig, W. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiology Reviews* **24,** 555–565 (2000).
7. Haffajee, A. D. & Socransky, S. S. Microbiology of periodontal diseases: Introduction. *Periodontology 2000* **38,** 9–12 (2005).
8. De Lillo, A. *et al.* Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. *Oral Microbiol. Immunol.* **21,** 61–68 (2006).
9. Colombo, A. P. V. *et al.* Comparisons of Subgingival Microbial Profiles of Refractory Periodontitis, Severe Periodontitis, and Periodontal Health Using the Human Oral Microbe Identification Microarray. *J. Periodontol.* **80,** 1421–1432 (2009).
10. Diaz, P. I. Microbial diversity and interactions in subgingival biofilm communities. in *Periodontal Disease* **15,** 17–40 (2011).
11. Haffajee, A. D. *et al.* Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. *J. Clin. Periodontol.* **25,** 346–353 (1998).
12. Gonçalves, L. F. H. *et al.* Levels of Selenomonas species in generalized aggressive periodontitis. *J. Periodontal Res.* **47,** 711–718 (2012).
13. Oliveira, R. R. D. S. *et al.* Levels of candidate periodontal pathogens in subgingival biofilm. *J. Dent. Res.* **95,** 711–718 (2016).
14. Costerton, J. W. *et al.* Biofilms, the customized microniche. *Journal of Bacteriology* **176,** 2137–2142 (1994).
15. Jhajharia, K., Mehta, L., Parolia, A. & Shetty, Kv. Biofilm in endodontics: A review. *J. Int. Soc. Prev. Community Dent.* **5,** 1 (2015).
16. Lewis, K. Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy* **45,** 999–

1007 (2001).

17. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. Extracellular DNA required for bacterial biofilm formation. *Science (80-. ).* **295,** 1487 (2002).

18. Socransky, S. S. & Haffajee, A. D. Dental biofilms: Difficult therapeutic targets. *Periodontol. 2000* **28,** 12–55 (2002).

19. Lowy, F. Bacterial Classification , Structure and Function. *Columbia Univ.* 1–6 (2009).

20. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284,** 1318–22 (1999).

21. Miller, M. B. & Bassler, B. L. Quorum Sensing in Bacteria. *Annu. Rev. Microbiol.* **55,** 165–199 (2001).

22. Bordi, C. & de Bentzmann, S. Hacking into bacterial biofilms: a new therapeutic challenge. *Ann Intensive Care* **1,** 19 (2011).

23. Haffajee, A. D., Teles, R. P. & Socransky, S. S. The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontology 2000* **42,** 219–258 (2006).

24. Takahashi, N. Oral microbiome metabolism: From 'who are they?' to 'what are they doing?' *Journal of Dental Research* **94,** 1628–1637 (2015).

25. Gibbons, R. J. & Houte, J. V. Bacterial Adherence in Oral Microbial Ecology. *Annu. Rev. Microbiol.* **29,** 19–42 (1975).

26. Avila, M., Ojcius, D. M. & Yilmaz, Ö. The Oral Microbiota: Living with a Permanent Guest. *DNA Cell Biol.* **28,** 405–411 (2009).

27. Socransky, S. S. & Manganiello, S. D. The Oral Microbiota of Man From Birth to Senility. *J. Periodontol.* **42,** 485–496 (1971).

28. Kolenbrander, P. E. & London, J. Adhere today, here tomorrow: Oral bacterial adherence. *Journal of Bacteriology* **175,** 3247–3252 (1993).

29. Loesche, W. J. Clinical and Microbiological Aspects of Chemotherapeutic Agents Used According to the Specific Plaque Hypothesis. *J. Dent. Res.* **58,** 2404–2412 (1979).

30. Theilade, E. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J. Clin. Periodontol.* **13,** 905–911 (1986).

31. Marsh, P. D. Microbial ecology of dental plaque and its significance in health and disease. *Advances in dental research* **8,** 263–271 (1994).

32. Rosier, B. T., De Jager, M., Zaura, E. & Krom, B. P. Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol* **4,** 92 (2014).

33. Pöllänen, M. T., Paino, A. & Ihalin, R. Environmental stimuli shape biofilm formation and the virulence of periodontal pathogens. *International Journal of Molecular Sciences* **14,** 17221–17237 (2013).

34. Listgarten, M. A., Mayo, H. E. & Tremblay, R. Development of dental plaque on epoxy resin crowns in man. A light and electron microscopic study. *J. Periodontol.* **46,** (1975).

35. Listgarten, M. A. & Shapiro, I. M. Fine structure and composition of coronal cementum in guinea-pig molars. *Arch. Oral Biol.* **19,** (1974).

36. Listgarten, M. . A. Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. *J. Periodontol.* **47,** 1–18 (1976).

37. Gmür, R., Strub, J. R. & Guggenheim, B. Prevalence of Bacteroides forsythus and Bacteroides gingivalis in subgingival plaque of prosthodontically treated patients on short recall. *J. Periodontal Res.* **24,** 113–120 (1989).

38. Ali, R. W., Skaug, N., Nilsen, R. & Bakken, V. Microbial associations of 4 putative periodontal pathogens in Sudanese adult periodontitis patients determined by DNA probe analysis. *J Periodontol* **65,** 1053–1057 (1994).

39. Kigure, T. *et al.* Distribution of Porphyromonas gingivalis and Treponema denticola in human subgingival plaque at different periodontal pocket depths examined by immunohistochemical methods. *J. Periodontal Res.* **30,** 332–341 (1995).

40. Simonson, L. G., Robinson, P. J., Pranger, R. J., Cohen, M. E. & Morton, H. E. Treponema denticola and Porphyromonas gingivalis as prognostic markers following periodontal treatment. *J. Periodontol.* **63,** 270–3 (1992).

41. Haffajee, A. D. The effect of SRP on the clinical and microbiological parameters of periodontal diseases. *J. Clin. Periodontol.* **24,** 324–334 (1997).

42. Socransky, S. S., Haffajee, A. D. & Dzink, J. L. Relationship of subgingival microbial complexes to clinical features at the sampled sites. *J. Clin. Periodontol.* **15,** 440–444 (1988).

43. Socransky, S. S. *et al.* 'Checkerboard' DNA-DNA hybridization. *Biotechniques* **17,** 788–792 (1994).

44. Grenier, D. Antagonistic effect of oral bacteria towards Treponema denticola. *J. Clin. Microbiol.* **34,** 1249–1252 (1996).

45. Kumar, P. S., Griffen, A. L., Moeschberger, M. L. & Leys, E. J. Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J. Clin. Microbiol.* **43,** 3944–3955 (2005).

46. Paster, B. J. *et al.* Bacterial Diversity in Human Subgingival Plaque. *J. Bacteriol.* **18312,** 3770–3783 (2001).

47. Pérez-Chaparro, P. J. *et al.* Newly identified pathogens associated with periodontitis: A systematic review. *Journal of Dental Research* **93,** 846–858 (2014).

48. Griffen, A. L. *et al.* Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* **6,** 1176–1185 (2012).

49. Colombo, A. P. V. *et al.* Comparisons of Subgingival Microbial Profiles of Refractory

Periodontitis, Severe Periodontitis, and Periodontal Health Using the Human Oral Microbe Identification Microarray. *J. Periodontol.* **80,** 1421–1432 (2009).

50. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory press*. *New York* (1989). doi:574.873224 1/1989

51. Teles, R., Teles, F., Frias-Lopez, J., Paster, B. & Haffajee, A. Lessons learned and unlearned in periodontal microbiology. *Periodontol. 2000* **62,** 95–162 (2013).

52. SLOTS, J. The predominant cultivable microflora of advanced periodontitis. *Eur. J. Oral Sci.* **85,** 114–121 (1977).

53. SLOTS, J. Microflora in the healthy gingival sulcus in man. *Eur. J. Oral Sci.* **85,** 247–254 (1977).

54. Socransky, S. S., Manganiello, A. D., Propas, D., Oram, V. & Van Houte, J. Bacteriological studies of developing supragingival dental plaque. *J. Periodontal Res.* **12,** 90–106 (1977).

55. Moore, W. E. C. *et al.* Bacteriology of experimental gingivitis in young adult humans. *Infect. Immun.* **38,** 651–667 (1982).

56. Tanner, A. C. R., Haffer, C., Bratthall, G. T., Visconti, R. A. & Socransky, S. S. A study of the bacteria associated with advancing periodontitis in man. *J. Clin. Periodontol.* **6,** 278–307 (1979).

57. Kumar, P. S. *et al.* New Bacterial Species Associated with Chronic Periodontitis. *J. Dent. Res.* **82,** 338–344 (2003).

58. Socransky, S. S. & Haffajee, A. D. Periodontal microbial ecology. *Periodontology 2000* **38,** 135–187 (2005).

59. Li, L. *et al.* Analyzing endodontic infections by deep coverage pyrosequencing. *J. Dent. Res.* **89,** 980–984 (2010).

60. Zaura, E., Keijser, B. J., Huse, S. M. & Crielaard, W. Defining the healthy ‘core microbiome’ of oral microbial communities. *BMC Microbiol.* **9,** (2009).

61. Teles, R. *et al.* Relationships Among Gingival Crevicular Fluid Biomarkers, Clinical Parameters of Periodontal Disease, and the Subgingival Microbiota. *J. Periodontol.* **81,** 89–98 (2010).

62. Socransky, S. S. *et al.* Use of checkerboard DNA-DNA hybridization to study complex microbial ecosystems. *Oral Microbiol. Immunol.* **19,** 352–362 (2004).

63. Kauppinen, S., Vester, B. & Wengel, J. Locked nucleic acid (LNA): High affinity targeting of RNA for diagnostics and therapeutics. *Drug Discovery Today: Technologies* **2,** 279–282 (2005).

64. Petersen, M. & Wengel, J. LNA: A versatile tool for therapeutics and genomics. *Trends in Biotechnology* **21,** 74–81 (2003).

65. Piao, X., Yan, Y., Yan, J. & Guan, Y. Enhanced recognition of non-complementary hybridization by single-LNA-modified oligonucleotide probes. *Anal. Bioanal. Chem.* **394,** 1637–1643 (2009).

66. Yan, J., Yuan, Y., Mu, R., Shang, H. & Guan, Y. LNA-modified isothermal oligonucleotide microarray for differentiating bacilli of similar origin. *J. Biosci.* **39,** 795–804 (2014).

67. Válóczi, A. *et al.* Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res.* **32,** e175 (2004).

68. Koshkin, A. A. *et al.* LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron* **54,** (1998).

69. Obika, S. *et al.* Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2'-O,4'-C- methyleneribonucleosides. *Tetrahedron Lett.* **39,** 5401–5404 (1998).

70. Braasch, D. a & Corey, D. R. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. *Chem. Biol.* **8,** 1–7 (2001).

71. Mouritzen, P. *et al.* Single nucleotide polymorphism genotyping using locked nucleic acid (LNA$^{TM}$). *Expert Review of Molecular Diagnostics* **3,** 27–38 (2003).

72. Tolstrup, N. *et al.* OligoDesign: Optimal design of LNA (locked nucleic acid) oligonucleotide capture probes for gene expression profiling. *Nucleic Acids Res.* **31,** 3758–3762 (2003).

73. Johnson, M. P., Haupt, L. M. & Griffiths, L. R. Locked nucleic acid (LNA) single nucleotide polymorphism (SNP) genotype analysis and validation using real-time PCR. *Nucleic Acids Res.* **32,** e55 (2004).

74. Ugozzoli, L. A., Latorra, D., Pucket, R., Arar, K. & Hamby, K. Real-time genotyping with oligonucleotide probes containing locked nucleic acids. *Anal. Biochem.* **324,** 143–152 (2004).

75. Kierzek, E. *et al.* The influence of locked nucleic acid residues on the thermodynamic properties of 2′-O-methyl RNA/RNA heteroduplexes. *Nucleic Acids Res.* **33,** (2005).

76. Camelo-Castillo, A. *et al.* Relationship between periodontitis-associated subgingival microbiota and clinical inflammation by 16S pyrosequencing. *J. Clin. Periodontol.* **42,** 1074–1082 (2015).

77. Pradhan-Palikhe, P. *et al.* Subgingival Bacterial Burden in Relation to Clinical and Radiographic Periodontal Parameters. *J. Periodontol.* **84,** 1809–1817 (2013).

78. Abusleme, L. *et al.* The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* **7,** 1016–1025 (2013).